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(71) Applicant *(for all designated States except US)*: CELLOMICS, INC. [US/US]; 635 William Pitt Way, Pittsburgh, PA 15238 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: JUNG, David, R. [US/US]; 122 Crescent Hills Road, Pittsburgh, PA 15235 (US). GOUGH, Albert, H. [US/US]; 2014 Louise Drive, Glenshaw, PA 15116 (US). TAYLOR, D., Lansing [US/US]; 910 Notre Dame Place, Pittsburgh, PA 15215 (US).

(74) Agent: HARPER, David, S.; McDonnell, Boehnen, Hulbert & Berghoff, Suite 3200, 300 South Wacker Drive, Chicago, IL 60606 (US).

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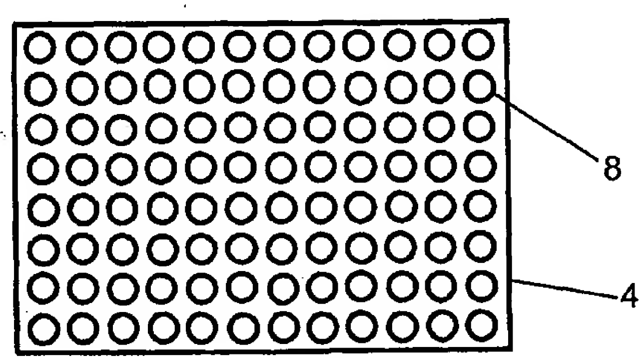
(54) Title: MINIATURIZED CELL ARRAY METHODS AND APPARATUS FOR CELL-BASED SCREENING

(57) Abstract

The present invention devices and methods for maximizing the number of wells that can be imaged at one time while still obtaining adequate pixel resolution in the image. This result has been achieved through the use of fluidic architectures that maximizes well density. The present invention thus provides a miniaturized microplate system with closed fluidic volumes that are internally supplied with fluid exchange, and with wells that are closely spaced to more rapidly detect spatially-resolved features of individual cells.



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Miniaturized Cell Array Methods and Apparatus for Cell-Based Screening

Related Applications

This application is a continuation-in-part of U.S. Provisional Application for Patent S/N 60/101,399, filed September 22, 1998, and U.S. Application for Patent S/N 08/865,341 filed on May 29, 1997, which are both hereby incorporated by reference in their entirety.

Field of the Invention

The present invention relates to methods and devices for cell-based high throughput and high biological content screening.

Background of the Invention

In the expanding arena of drug discovery and combinatorial chemistry to generate candidate compounds, it would be very useful to be able to rapidly screen a large number of substances, via a high throughput screen, for their physiological impact on animals and humans. Before testing the efficacy of a "partially qualified" drug candidate on animals, the drug could first be screened for its biological activity and potential toxicity with living cells. The physiological response to the drug candidate could then be anticipated from the results of these cell screens.

Traditionally, "lead compounds" have moved quickly to extensive animal studies which are both time-consuming and costly. Furthermore, extensive drug testing in animals is becoming less culturally acceptable. Screening drug candidates according to their interaction with living cells, prior to animal studies, can reduce the number of animals required in subsequent drug screening processes by eliminating some drug candidates before going to animal trials. However, manipulation and analysis of drug-cell interactions using current methods does not allow for both high throughput and high biological content screening, due to the small number of cells and compounds that can be analyzed in a given period of time, the cumbersome methods required for compound delivery, and the large volumes of compounds required for testing.

High throughput screening of nucleic acids and polypeptides has been achieved using DNA chip technologies. In typical DNA analysis methods, DNA sequences of 10 to 14

substrate pairs are attached in defined locations (or spots), up to tens of thousands in number, on a small glass plate. (U.S. Patent No. 5,556,752, hereby incorporated by reference). This creates an array of spots of DNA on a given glass plate. The location of a spot in the array provides an address for later reference to each spot of DNA. The DNA sequences are then hybridized with complementary DNA sequences labeled with fluorescent molecules. Signals from each address on the array are detected when the fluorescent molecules attached to the hybridizing nucleic acid sequences fluoresce in the presence of light. These devices have been used to provide high throughput screening of DNA sequences in drug discovery efforts and in the human genome sequencing project. Similarly, protein sequences of varying amino acid lengths have been attached in discrete spots as an array on a glass plate. (U.S. Patent 5,143,854, incorporated by reference herein).

The information provided by an array of either nucleic acids or amino acids bound to glass plates is limited according to their underlying "languages". For example, DNA sequences have a language of only four nucleic acids and proteins have a language of about 20 amino acids. In contrast, a living cell, which comprises a complex organization of biological components, has a vast "language" with a concomitant multitude of potential interactions with a variety of substances, such as DNA, RNA, cell surface proteins, intracellular proteins and the like. Because a typical target for drug action is with and within the cells of the body, cells themselves provide an extremely useful screening tool in drug discovery when combined with sensitive detection reagents. It thus would be most useful to have a high throughput, high content screening device to provide high content spatial information at the cellular and subcellular level as well as temporal information about changes in physiological, biochemical and molecular activities.

Microarrays of cells

Methods have been described for making micro-arrays of a single cell type on a common substrate for other applications. One example of such methods is photochemical resist-photolithograpy (Mrksich and Whitesides, Ann. Rev. Biophys. Biomol. Struct. 25:55-78, 1996), in which a glass plate is uniformly coated with a photoresist and a photo mask is placed over the photoresist coating to define the "array" or pattern desired. Upon exposure to light, the photoresist in the unmasked areas is removed. The entire photolithographically defined surface is uniformly coated with a hydrophobic substance, such as an organosilane,

that binds both to the areas of exposed glass and the areas covered with the photoresist. The photoresist is then stripped from the glass surface, exposing an array of spots of exposed glass. The glass plate then is washed with an organosilane having terminal hydrophilic groups or chemically reactable groups such as amino groups. The hydrophilic organosilane binds to the spots of exposed glass with the resulting glass plate having an array of hydrophilic or reactable spots (located in the areas of the original photoresist) across a hydrophobic surface. The array of spots of hydrophilic groups provides a substrate for non-specific and non-covalent binding of certain cells, including those of neuronal origin (Kleinfeld et al., J. Neurosci. 8:4098-4120, 1988).

In another method based on specific yet non-covalent interactions, stamping is used to produce a gold surface coated with protein adsorptive alkanethiol. (U.S. Patent No. 5,776,748; Singhvi et al., Science 264:696-698, 1994). The bare gold surface is then coated with polyethylene-glycol-terminated alkanethiols that resist protein adsorption. After exposure of the entire surface to laminin, a cell-binding protein found in the extracellular matrix, living hepatocytes attach uniformly to, and grow upon, the laminin coated islands (Singhvi et al. 1994). An elaboration involving strong, but non-covalent, metal chelation has been used to coat gold surfaces with patterns of specific proteins (Sigal et al., Anal. Chem. 68:490-497, 1996). In this case, the gold surface is patterned with alkanethiols terminated with nitriloacetic acid. Bare regions of gold are coated with tri(ethyleneglycol) to reduce protein adsorption. After adding Ni^{2+}, the specific adsorption of five histidine-tagged proteins is found to be kinetically stable.

More specific single cell-type binding can be achieved by chemically crosslinking specific molecules, such as proteins, to reactable sites on the patterned substrate. (Aplin and Hughes, Analyt. Biochem. 113:144-148, 1981). Another elaboration of substrate patterning optically creates an array of reactable spots. A glass plate is washed with an organosilane that chemisorbs to the glass to coat the glass. The organosilane coating is irradiated by deep UV light through an optical mask that defines a pattern of an array. The irradiation cleaves the Si-C bond to form a reactive Si radical. Reaction with water causes the Si radicals to form polar silanol groups. The polar silanol groups constitute spots on the array and are further modified to couple other reactable molecules to the spots, as disclosed in U.S. Patent No. 5,324,591, incorporated by reference herein. For example, a silane containing a biologically functional group such as a free amino moiety can be reacted with the silanol

groups. The free amino groups can then be used as sites of covalent attachment for biomolecules such as proteins, nucleic acids, carbohydrates, and lipids. The non-patterned covalent attachment of a lectin, known to interact with the surface of cells, to a glass substrate through reactive amino groups has been demonstrated (Aplin & Hughes, 1981). The optical method of forming a micro-array of a single cell type on a support requires fewer steps and is faster than the photoresist method, (i.e., only two steps), but it requires the use of high intensity ultraviolet light from an expensive light source.

In all of these methods, the result is a micro-array of a single cell type, since the biochemically specific molecules are bound to the micro-patterned chemical array uniformly. In the photoresist method, cells bind to the array of hydrophilic spots and/or specific molecules attached to the spots which, in turn, bind cells. Thus cells bind to all spots in the array in the same manner. In the optical method, cells bind to the array of spots of free amino groups by adhesion. There is little or no differentiation between the spots of free amino groups. Again, cells adhere to all spots in the same manner, and thus only a single type of cell interaction can be studied with these cell arrays because each spot on the array is essentially the same as another. Such cell arrays are inflexible in their utility as tools for studying a specific variety of cells in a single sample or a specific variety of cell interactions. Thus, there exists a need for arrays of multiple cell types on a common substrate , in order to increase the number of cell types and specific cell interactions that can be analyzed simultaneously, as well as methods of producing these micro-arrays of multiple cell types on a common substrate, in order to provide for high throughput and high biological content screening of cells.

Optical reading of cell physiology

Performing a high throughput screen on many thousands of compounds requires parallel handling and processing of many compounds and assay component reagents. Standard high throughput screens use homogeneous mixtures of compounds and biological reagents along with some indicator compound, loaded into arrays of wells in standard microplates with 96 or 384 wells. (Kahl et al., J. Biomol. Scr. 2:33-40, 1997). The signal measured from each well, either fluorescence emission, optical density, or radioactivity, integrates the signal from all the material in the well giving an overall population average of

all the molecules in the well. This type of assay is commonly referred to as a homogeneous assay.

U.S. Patent No. 5,581,487 describes an imaging plate reader that uses a CCD detector (charge-coupled optical detector) to image the whole area of a 96 well plate. The image is analyzed to calculate the total fluorescence per well for homogeneous assays.

Schroeder and Neagle describe a system that uses low angle laser scanning illumination and a mask to selectively excite fluorescence within approximately 200 microns of the bottoms of the wells in standard 96 well plates in order to reduce background when imaging cell monolayers. (J. Biomol. Scr. 1:75-80, 1996). This system uses a CCD camera to image the whole area of the plate bottom. Although this system measures signals originating from a cell monolayer at the bottom of the well, the signal measured is averaged over the area of the well and is therefore still considered a homogeneous measurement, since it is an average response of a population of cells. The image is analyzed to calculate the total fluorescence per well for cell-based homogeneous assays.

Proffitt et. al. (Cytometry 24:204-213, 1996) describe a semi-automated fluorescence digital imaging system for quantifying relative cell numbers *in situ*, where the cells have been pretreated with fluorescein diacetate (FDA). The system utilizes a variety of tissue culture plate formats, particularly 96-well microplates. The system consists of an epifluorescence inverted microscope with a motorized stage, video camera, image intensifier, and a microcomputer with a PC-Vision digitizer. Turbo Pascal software controls the stage and scans the plate taking multiple images per well. The software calculates total fluorescence per well, provides for daily calibration, and configures for a variety of tissue culture plate formats. Thresholding of digital images and the use of reagents that fluoresce only when taken up by living cells are used to reduce background fluorescence without removing excess fluorescent reagent..

A variety of methods have been developed to image fluorescent cells with a microscope and extract information about the spatial distribution and temporal changes occurring in these cells. A recent article describes many of these methods and their applications (Taylor et al., Am. Scientist 80:322-335, 1992). These methods have been designed and optimized for the preparation of small numbers of specimens for high spatial and temporal resolution imaging measurements of distribution, amount and biochemical environment of the fluorescent reporter molecules in the cells.

Treating cells with dyes and fluorescent reagents, imaging the cells, and engineering the cells to produce a fluorescent reporter molecule, such as modified green fluorescent protein (GFP), are useful detection methods (Wang et al., In Methods in Cell Biology, New York, Alan R. Liss, 29:1-12, 1989). The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has an excitation maximum at 395 nm, an emission maximum at 510 nm and does not require an exogenous factor. Uses of GFP for the study of gene expression and protein localization are discussed in Chalfie et al., Science 263:802-805, 1994. Some properties of wild-type GFP are disclosed by Morise et al. (Biochemistry 13:2656-2662, 1974), and Ward *et al.* (Photochem. Photobiol. 31:611-615, 1980). An article by Rizzuto *et al.* (Nature 358:325-327, 1992) discusses the use of wild-type GFP as a tool for visualizing subcellular organelles in cells. Kaether and Gerdes (FEBS Letters 369:267-271, 1995) report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng (FEBS Letters 369:331-334, 1995), while GFP expression in Drosophila embryos is described by Davis et al. (Dev. Biology 170:726-729, 1995). U. S. Patent No. 5,491,084, incorporated by reference herein, discloses expression of GFP from *Aequorea victoria* in cells as a reporter molecule fused to another protein of interest. Mutants of GFP have been prepared and used in several biological systems. (Hasselhoff et al., Proc. Natl. Acad. Sci. 94:2122-2127, 1997; Brejc et al., Proc. Natl. Acad. Sci. 94:2306-2311, 1997; Cheng et al., Nature Biotech. 14:606-609, 1996; Heim and Tsien, Curr. Biol. 6:178-192, 1996; Ehrig et al., FEBS Letters 367:163-166, 1995).

The ARRAYSCAN™ System, as developed by Cellomics, Inc. (U.S. Patent Application Serial Nos. 08/810,983 filed February 27, 1997 and 09/031,271 filed February 27, 1998) is an optical system for determining the distribution, environment, or activity of luminescently labeled reporter molecules in cells for the purpose of screening large numbers of compounds for specific biological activity. The ARRAYSCAN™ System involves providing cells containing luminescent reporter molecules in an array of locations and scanning numerous cells in each location, converting the optical information into digital data, and utilizing the digital data to determine the distribution, environment or activity of the luminescently labeled reporter molecules in the cells. The ARRAYSCAN™ System includes apparatus and computerized method for processing, displaying and storing the data, thus augmenting drug discovery by providing high content cell-based screening, as well as

combined high throughput and high content cell-based screening, in a large microplate format.

Microfluidics

Efficient delivery of solutions to an array of cells attached to a solid substrate is facilitated by a microfluidic system. Methods and apparatus have been described for the precise handling of small liquid samples for ink delivery (U.S. Patent No. 5,233,369; U.S. Patent No. 5,486,855; U.S. Patent No. 5,502,467), biosample aspiration (U.S. Patent No. 4,982,739), reagent storage and delivery (U.S. Patent No. 5,031,797), and partitioned microelectronic and fluidic device array for clinical diagnostics and chemical synthesis (U.S. Patent No. 5,585,069). In addition, methods and apparatus have been described for the formation of microchannels in solid substrates that can be used to direct small liquid samples along the surface (U.S. Patent No. 5,571,410; U.S. Patent No. 5,500,071; U.S. Patent No. 4,344,816,).

For purposes of integrated high throughput and high content cell based screening, particularly for live-cell imaging, an optimal microfluidic device would comprise a fluidic architecture that permits the closest possible well spacing (i.e.: highest possible well density), wherein the fluidic architecture is integrated with the cell array substrate to permit efficient fluid delivery to the cells, and eliminating the need for pipetting fluids in and out of wells. Such optimal microfluidic devices would be advantageous for cell arrays with sub-millimeter inter-well distances because it is unwieldy, if not impossible, to pipette fluids with such a high degree of spatial resolution and accuracy. Furthermore, such integrated devices could be directly used for cell based screening, without the need to remove the cell substrate from the fluidic architecture for imaging the cells.

An optimal microfluidic device for cell based screening might further comprise a closed chamber to permit environmental control of the cells, and preferably would not directly expose the cells to electro-kinetic forces, which may affect the physiology of the cells on the substrate. For example, electrohydrodynamic pumping is less effective with polar solvents (Marc Madou, Fundamentals of Microfabrication, CRC Press, Boca Raton, 1997, p. 433). Electro-osmosis is typically accompanied by some degree of electrophoretic separation of charged medium components, such as proteins.

U.S. Patent No. 5,603,351 ('the 351 patent') describes a microfluidic device that uses a multilevel design consisting of two upper levels with channels and a bottom level with reaction wells. However, this device is not designed for use in cell based screening. The '351 patent does not disclose a substrate containing cells or cell binding sites. The disclosed microfluidic network is designed to allow two or more reagents to be combined in a reaction well, as opposed to an optimal cell screening microfluidic system that allows living cells cultured on the well bottoms to be exposed in serial fashion to two or more different fluids. The '351 patent discloses a device with the wells etched into the substrate at a maximal well density of 50 wells/inch^2. Furthermore, the substrate must be detached from the fluidic array for incubation and/or analysis. Finally, the '351 patent discloses a system of electrically-controlled electrohydrodynamic valves within the matrix of the wells that are less effective with aqueous media used in cell culture, and also may limit the degree of close-spacing between wells in the array of wells.

U.S. Patent No. 5,655,560 discloses a clog-free valving system, comprising a fluid distribution system with multiple inputs and multiple outputs incorporating a crossed array of microchannels connected vertically at crossing points by teflon valves. However, this patent does not disclose a substrate containing a cell array, nor an integrated fluidic device in combination with the substrate, nor a well density that is optimal for cell-based screening.

U.S. Patent 5,900,130 (the '130' patent) describes the active, electronic control of fluid movement in an interconnected capillary structure. This patent does not teach a fluidic architecture that maximizes the area of the cell substrate that can be occupied by cell binding sites. Nor does this patent disclose a substrate containing a cell array, nor an integrated fluidic device in combination with the substrate. Furthermore, the patent only teaches the control of fluid flow by application of an electric field to the device.

U.S. Patent 5,910,287 describes multi-well plastic plates for fluorescence measurements of biological and biochemical samples, including cells, limited to plates with greater than 864 wells. This patent does not describe a microfluidic device with a fluidic architecture integrated with the cell array substrate. Nor does the patent disclose a closed chamber to permit environmental control of the cells on the substrate.

Thus, none of these prior microfluidic devices provide a fluidic architecture that permits the closest possible well spacing (i.e.: highest possible well density), wherein the fluidic architecture is integrated with the cell array substrate to permit efficient fluid delivery

to the cells, and thus eliminating the need for pipetting fluids in and out of wells. Furthermore, prior microfluidic devices which comprise an array of wells use electrically-controlled electrohydrodynamic valves within the matrix of the wells that would be less effective if used with aqueous media for cell culture, and which limit the well density.

While the above advances in cell array, optical cell physiology reading, and microfluidic technologies provide supporting technologies that can be applied to improved high throughput and high content cell-based screening, there remains a need in the art for integrated devices and methods that further decrease the amount of time necessary for such screening, as well as for devices and methods that further improve the ability to conduct high throughput and high content cell-based screening and the ability to flexibly and rapidly switch from one to the other. In particular, devices and methods that maximize the well density, thereby increasing the number of wells that can be imaged in at one time, and thus greatly increasing the throughput of a screen while maintaining adequate resolution of the image, would be very advantageous.

The drug discovery industry already uses 96- and 384-well microplates and is in transition towards the use of 1536-well plates. However, further increases in well density using prior technology are unlikely because of the great difficulty of pipetting liquids in and out of very small diameter wells.

Summary of the Invention

The present invention fulfills the need in the art for devices and methods that decrease the amount of time necessary to conduct cell-based screening, and specifically combines the ability to conduct high throughput and high content cell-based screening and to flexibly and rapidly switch from one to the other The invention provides devices and methods for maximizing the number of wells that can be imaged at one time while still obtaining adequate pixel resolution in the image. This result has been achieved through the use of fluidic architectures that maximizes well density. The present invention thus provides a miniaturized microplate system with closed fluidic volumes that are internally supplied with fluid exchange, and with wells that are closely spaced to more rapidly detect spatially-resolved features of individual cells.

In one aspect, the present invention provides a cassette for cell screening, comprising a substrate having a surface containing a plurality of cell binding locations; a fluid delivery

system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the substrate, wherein the multi-level chamber comprises a crossed array of microfluidic input channels and output channels and a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels; and a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location, and wherein the wells are present at a density of at least about 20 wells per square centimeter.

In another aspect, the present invention provides a cassette for cell screening comprising

a. a substrate having a surface, wherein the surface contains a plurality of cell binding locations;

b. a fluid delivery system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the substrate, wherein the multi-level chamber comprises

i. a crossed array of microfluidic input channels and output channels, wherein each well is in fluid connection with one or more input channels and one or more output channels;

ii. a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels;

iii. one or more input manifolds in fluid connection with the microfluidic input channels;

iv. one or more output manifolds in fluid connection with the microfluidic output channels;

v. at least one source receptacle in fluid connection with the one or more input manifolds; and

vi. at least one waste receptacle in fluid connection with one or more output manifolds; and

c. a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location.

In preferred embodiments, both of the above cassettes further comprise a pump to control fluid flow within the microfluidic device; a temperature controller of the substrate and/or a controller to regulate oxygen and carbon dioxide partial pressures.

In another aspect, the present invention provides an improved method for diffusion control in a cassette, wherein the improvement comprises constantly applying a passive restoring force to valves located within microfluidic channels of the cassette.

In a still further aspect, the present invention provides a method for cell screening, comprising

a) providing an array of locations which contain multiple cells;

b) providing an optical system to obtain images of the array of locations;

c) serially imaging sub-arrays of the array of locations; and

d) acquiring data from each of the sub-arrays in parallel.

In a preferred embodiment, the array of locations is provided as a cassette, such as those disclosed above.

Among other uses, the devices and methods of the present invention are ideal for high content and/or high throughput cell-based screening. The device of the invention is also ideally suited as a cell support system for a hand-held diagnostic device (i.e.: a miniaturized imaging cell-based assay system). The smaller format and sealed containment of the present device enables its use in a rugged, portable system. There is a great economic advantage from the use of higher density plates. There is a further economic advantage in faster imaging of sub-arrays when using a high density plate; this advantage is not fully realized if the distances between adjacent wells are not minimized. The present invention provides both of these advantages.

Brief Description of the Figures

Figure 1A is a top view of a small substrate micro-patterned chemical array.

Figure 1B is a top view of a large substrate micro-patterned chemical array.

Figure 2 diagrams a method of producing a micro-patterned chemical array on a substrate.

Figure 3A is a photograph showing fibroblastic cell growth on a surface patterned chip, attached to a micro-patterned chemical array and labeled with two fluorescent probes.

Figure 3B is a photograph showing fibroblastic cell growth in spotted patterns, attached to a micro-patterned chemical array and labeled with two fluorescent probes.

Figure 4 is a diagram of the cassette which is the combination of the micro-array of multiple cell types top and chamber bottom.

Figure 5 is a diagram of a chamber that has nanofabricated input channels to address "wells" in the non-uniform micro-patterned array of cells.

Figure 6 is a diagram of a chamber with no channels.

Figure 7A is an overhead diagram of a chamber with microfluidic channels etched onto the substrate.

Figure 7B is a side view diagram of a chamber with microfluidic channels etched onto the substrate.

Figure 8A is an overhead diagram of a chamber where the microfluidic channels and wells are formed from a raised matrix of a material stamped onto the fluid delivery chamber.

Figure 8B is a side view diagram of a chamber where the microfluidic channels and wells are formed from a raised matrix of a material stamped onto the fluid delivery chamber.

Figure 9 is a diagram of a chamber where each well is addressed by a channel originating from one side of the chamber.

Figure 10 is a diagram of a chamber where the wells are addressed by channels originating from two sides of the chamber.

Figure 11 is a diagram of a chamber where the microfluidic switches are controlled by light, heat or other mechanical means.

Figure 12 is a diagram of the luminescence reader instrument.

Figure 13 is a diagram of one embodiment of the luminescence reader instrument optical system.

Figure 14A is a flow chart providing an overview of the cell screening method.

Figure 14B is a Macro (High Throughput Mode) Processing flow chart.

Figure 14C is a Micro (High Content Mode) Processing flow chart.

Figure 15 is a diagram of the integrated cell screening system.

Figure 16 is a photograph of the user interface of the luminescence reader instrument.

Figure 17A is a photograph showing lymphoid cells nonspecifically attached to an unmodified substrate.

Figure 17B is a photograph showing lymphoid cells nonspecifically attached to an IgM-coated substrate.

Figure 17C is a photograph showing lymphoid cells specifically bound to a whole anti-serum-coated substrate.

Figure 18A is a photographic image from High Throughput Mode of luminescence reader instrument identifying "hits".

Figure 18B is a series of photographic images showing the high content mode identifying high content biological information.

Figure 19 is a photographic image showing the display of cell data gathered from the high content mode.

Figure 20 depicts the optimization of cell imaging by simultaneous imaging of a sub-array of cell binding locations of the whole array. There is a one to one correspondence between the array of cell binding locations on the substrate, the array of fluidic locations on the chamber, and the array of wells formed by the joining of the two.

Figure 21 exemplifies an 8 x 8 array (64 wells, 128 channels) of the microfluidic device wherein each fluidic location is provided with a separate input and output channel, yielding $2n^2$ channels for an $n \times n$ array.

Figure 22 depicts the microfluidic device of Figure 21 wherein $m + n^2$ valves or pumps are utilized to control the flow of m liquid or vapor mixtures to an 8 x 8 array.

Figure 23 depicts an embodiment of the microfluidic device wherein the fluid delivery system consists of a crossed, nonintersecting array of input and output channels, and horizontal connecting channels utilizing multiple levels, and wherein the two layers lie in the same plane of the well layer.

Figure 24 depicts an end view and side view of the embodiment of Figure 23.

Figure 25 depicts an embodiment of the microfluidic device wherein the fluid delivery system consists of a crossed, nonintersecting array of input and output channels, and vertical connecting channels utilizing multiple levels, and wherein the two layers lie in a plane above that of the well layer.

Figure 26 depicts an end view and side view of the embodiment of Figure 25.

Figure 27 shows an embodiment of the microfluidic device wherein compounds are multiplexed to a single fluidic location by using m positive pressure source reservoirs or pumps, a valve-less manifold, and valve-free waste reservoir at atmospheric pressure.

Figure 28 shows an embodiment of the microfluidic device wherein compounds are multiplexed to a single fluidic location by using m positive pressure source reservoirs or

pumps, a valved manifold, or a negative pressure waste reservoir or pump with *m* valves connected to *m* source reservoirs at atmospheric pressure and valve-free waste reservoir at atmospheric pressure.

Figure 29 shows an embodiment of the microfluidic device wherein the array of *m* positive pressure source reservoirs or pumps is multiplexed to an *n x n* crossed channel array of fluidic locations by means of *1 x n* input and *n x 1* output valve manifolds.

Figure 30 shows an embodiment of the microfluidic device with *m* reservoirs at atmospheric pressure and a negative pressure waste reservoir by means of *1 x n* input and *n x 1* output valve manifolds, where the negative pressure reservoir may be either mechanically or thermodynamically pumped.

Figure 31 shows an embodiment a dual-pumped system, in which there is only one waste negative pressure reservoir that must be actuated in coordinated fashion with the actuation of any particular positive pressure source reservoir. This negative pressure reservoir may function by either mechanical or thermodynamic (e.g., a capillary action pump) means. For the capillary action pump, the "actuation" of the pump is acheived by means of valves.

Figure 32 shows how the pumping and valving scheme of Figure 29, employing positive pressure source reservoirs, can be extended to work with a crossed array of *n* row channels and *2n* column channels (*n* ordinary column channels plus *n* rinse column channels). This requires a *2n x 1* output valve manifold. Similarly, the scheme of Figure 30, employing a negative pressure waste reservoir, can be extended to work with an array incorporating rinse channels.

Figure 33 depicts an embodiment of the microfluidic device wherein <u>every</u> column channel *k* has an associated parallel rinse channel *k**.

Figure 34 depicts an embodiment of the microfluidic device wherein the fluid delivery system consists of a crossed, nonintersecting array of input and output channels with vertical connecting channels, utilizing multiple levels, wherein the two layers lie in a plane above that of the well layer and <u>every</u> column channel *k* has an associated parallel rinse channel *k**.

Figure 35 shows, in a case of fluid flow through well *(j,k)*, an example of a method to rinse a segment of liquid or vapor from row channel *j* and into rinse channel *k** , where *k** is immediately adjacent to column channel *k*.

Figure 36 depicts a method of diffusion control using "normally-closed" check valves, comprising a valve element and a valve seat, between every pair of adjacent vias that connect the channels to the fluidic locations.

Figure 37 depicts an embodiment wherein an externally-applied magnetic field gradient induces a restoring force on valve elements and valve seat that incorporate a magnetic material.

Figure 38 shows an embodiment of a shaped valve seat wherein diffusion past the ball is stopped when the externally-applied restoring force presses the ball to the left into a round opening in the valve seat.

Figure 39 shows an embodiment that allows a single magnetic field gradient applied to the whole microfluidic device to induce force on both a set of ball valves of the input channels and a set of ball valves of the output channels

Figure 40 shows one embodiment of the pumping and valving schemes of this invention as it would appear with all the pumps and valves on-board the cassette.

Figure 41 shows an embodiment, wherein the pumps are on-board and utilize electrically-driven flow, where the electric fields are limited to regions external to the matrix of the array of wells, and minimal or no electric field gradients are applied across any of the wells in which live cells are present.

GUIDE TO THE FIGURES

001	fluidic location
002	array of fluidic locations, optionally with depressions
003	sub-array
004	substrate
008	cell binding location
009	chemical modification of the cell binding locations
010	cells arrayed on the cell binding locations
012	chamber
013	depression formed at fluidic location
014	input channel
016	output channel

018	cassette
020	spacer supports
024	micro-capillary tubes
028	raised reservoir
030	first channel
032	second channel
036	plug
040	array of fluidic locations
044	luminescence reader instrument
048	first storage compartment
050	first robotic arm
052	second robotic arm
054	second storage compartment
056	computer
060	Optics
064	computer controlled x-y-z stage
068	computer-controlled rotating nosepiece
070	low-magnification objective
072	high-magnification objective
074	while-light source lamp
076	excitation filter wheel
078	dichroic filter system
080	emission filters
082	detector (e.g., cooled CCD)
086	computer screen
090	database
140	lateral space between wells
160	horizontal connecting channels
180	vertical connecting channels
200	switchable positive pressure source reservoirs or pumps

220	source reservoirs at atmospheric pressure
240	m x 1 valve-less manifold
300	waste reservoirs
320	switchable negative pressure waste reservoirs or pumps
340	thermodynamically-pumped waste reservoir
400	1 x n valve manifold
410	n x 1 valve manifold
420	2n x 1 valve manifold
500	Check Valve
520	Valve Element
540	Valve Seat
560	magnetic (B) field gradient
580	restoring force on magnetic ball check valve
600	Bead
620	Magnetic Field
700	Fill ports
710	Channels
720	Valves
730	Reservoirs
740	Electrokinetic pump
760	Electrode 1
780	Electrode 2

Detailed Description Of The Preferred Embodiments

All patents, patent applications, and references cited herein are incorporated by reference in their entirety.

As used herein the term "well" is defined as the volume space created by the mating of one fluidic location of the chamber with one cell binding location on the substrate. The volume space may be created by a depression on the chamber corresponding to the fluidic location, by a depression on the substrate corresponding to the cell binding location, by a

spacer support that separates the chamber and the substrate (wherein there is no requirement for a depression in the substrate of the chamber), a combination of any of these, or any other suitable method for creating a volume space between the fluidic location and the cell binding location.

As used herein, the term "cell binding location" refers to a discrete location on the substrate that comprises a plurality of cell binding sites capable of binding cells. The substrate may be derivatized to create cell binding sites, or the cell binding sites may naturally be present on the substrate. The plurality of cell binding sites within an individual cell binding location may be capable of binding only a single cell type, or may be capable of binding more than one cell type. Different cell binding locations on the same substrate may be identical or different in the type of cells that they are capable of binding.

The term "fluidic location" as used herein refers to a discrete location on the chamber that is the site of fluid delivery to and/or removal from the cell binding location. The fluidic location may comprise a depression, such as an etched domain, a raised reservoir, or any other type of depression. Alternatively, the fluidic location may be flat, such as the terminus of an input and/or output channel, or the terminus of a via that is in fluid connection with an input and/or output channel.

As used herein, the term "cassette" means the combination of the substrate and the chamber. The combination can be either modular (more than one piece), providing a re-usable chamber and a disposable substrate, or non-modular (single piece), to limit any potential interwell leaking.

As used herein, the term "on-board" means integral with the cassette.

As used herein, the term "integrated fluidics" means that a microfluidic device comprises both a substrate surface for cell binding and a chamber.

As used herein, the term "chamber" means a fluid delivery system comprising microfluidic channels and fluidic locations, which serves as a specialized substrate cover.

As used herein, the term "switchable pump" refers to a pump, including but not limited to a syringe pump, a pressure-controlled vessel with a valve, or an electrokinetic pump that is employed externally (with the optional use of electrical shielding) with respect to the matrix of the array of wells, so that no harmful electric field effects are experienced by the cells.

As used herein, the term "sub-array" means any contiguous sub-section of wells in the entire array of wells, typically in a format corresponding to the shape of an imaging detector array, such as a CCD (charge coupled device detector).

As used herein the term "matrix of the cell array" means the space defined by the imaginary parallelpiped (i.e., typically a cubic or rectangular box) that would fit around the entire set of wells in the array of the cassette.

As used herein, the term "assay components" refers to any component that would be added to a cell screening assay, including but not limited to reagents, cells, test compounds, media, antibodies, luminescent and reporters.

As used herein, the term "luminescent" encompasses any type of light emission, including but not limited to luminescence, fluorescence, and chemiluminescence.

In one aspect, the present invention teaches a method of making a micro-array of multiple cell types on a common substrate. As defined herein, a micro-array of multiple cell types refers to an array of cells on a substrate that are not distributed in a single uniform coating on the support surface, but rather in a non-uniform fashion such that each "cell binding location" or groups of cell binding locations on the substrate may be unique in its cell binding selectivity.

The method of making a micro-array of multiple cell types comprises preparing a micro-patterned chemical array (also referred to herein as a chemically modified array of cell binding locations), chemically modifying the array non-uniformly, and binding cells to the non-uniform chemical array.

In a preferred embodiment, a micro-patterned chemical array comprises a substrate **4** which is treated to produce a hydrophobic surface across which are dispersed at regular intervals hydrophilic spots or "cell binding locations" **8**. **(Figure 1A-1B)**. The substrate can be a glass, plastic, or silicon wafer, such as a conventional light microscope coverslip, but can also be made of any other suitable material to provide a substrate. As describe previously, the term " cell binding locations" is used to describe a specific spot on the substrate, and does not require any particular depth. The surface of the substrate **4** is preferably about 2 cm by 3 cm, but can be larger or smaller. In a preferred embodiment, the cell binding locations **8** of the micro-patterned chemical array contain reactable functional groups such as, but not limited to, amino hydroxyl, sulfhydryl or carboxyl groups that can

bind to cells non-specifically or be further chemically modified to bind molecules that bind cells specifically.

Chemically modified cell binding locations are produced by specific chemical modifications of the cell binding locations on the substrate. The cell binding locations may comprise a variety of different cell binding molecules that permit attachment and growth of different types of cells in the cell binding locations, or may permit attachment of only a single cell type. The hydrophobic domains surrounding the cell binding locations on the substrate do not support the attachment and growth of the cells.

In one embodiment, a micro-array of multiple cell types is made by coating a glass wafer via chemisorbance with a layer of a substance having reactable functional groups such as amino groups. In a preferred embodiment, an aminosilane such as 3-amino propyltrimethoxysilane (APTS) or N-(2-aminoethyl-3-aminopropyl)trimethoxysilane (EDA) is used, but other reactable substances may be used. Following this first step, due to the presence of the reactable functional groups, the entire surface of the coated glass wafer is hydrophilic.

Secondly, a micro-patterning reaction is carried out where drops containing a substance having photo-cleavable or chemically removable amino protecting groups are placed in a micro-pattern of discrete locations on the aminosilane coated glass wafer. In one embodiment the pattern comprises a rectangular or square array, but any suitable discrete pattern, may be used (such as, but not limited to, triangular or circular). In one embodiment, the drops range in volume from 1 nanoliter (nl) to 1000 nl. In a preferred embodiment the drops range from 250-500 nl in volume. Suitable photochemically removable amino protecting substances include, but are not limited to 4-bromomethyl-3-nitrobenzene , 1-(4,5-dimethoxy-2-nitrophenyl)-ethyl (DMNPE) and butyloxycarbonyl. In one embodiment, the patterning reaction is carried out for 1 to 100 minutes at temperatures ranging from ambient temperature to 37°C, using reagent concentrations of between 1 micromolar (uM) and 1000 uM. In a preferred embodiment, the reaction is carried out at 37°C for 60 minutes using a reagent concentration of 500 uM.

The drops may be placed onto the aminosilane coated glass wafer via conventional ink-jet technology. (U.S Patent No. 5,233,369; U.S. Patent No. 5,486,855). Alternatively, an array of pins, defined herein as tapered rods that can transfer between 1 nl and 1000 nl of fluid, is dipped into a bath of the amino protecting substance to produce drops of the

protecting substance on their ends. The pins are then contacted with the glass wafer to transfer the drops thereto. In another embodiment, an array of capillary tubes made of glass or plastic, as described in U.S Patent Nos. 5,567,294 and 5,527,673, (both herein incorporated by reference), containing the amino protecting substance is contacted with the glass wafer to transfer the droplets to the surface. Thus, the glass wafer is micro-patterned with an array of spots or cell binding locations that contain protected amino groups on a hydrophobic surface (**Figure 2A-B**).

Third, a hydrophobic substance reactive with unprotected amino groups is washed over the glass wafer. The hydrophobic substance can be a fatty acid or an alkyl iodide, or any other suitable structure. Certain conditions for such a derivatization of glass can be found in Prime and Whitesides, Science 252:1164-1167, 1991, Lopez et al., J. Am. Chem. Soc. 115:5877-5878, 1993, and Mrksich and Whitesides, Ann. Rev. Biophys. Biomol. Struct. 25:55-78, 1996. The fatty acid or alkyl iodide reacts with the unprotected amino groups and covalently attaches thereto, and the amino groups are now hydrophobic due to the fatty acid or alkyl iodide group. The resulting modified array of cell binding locations **9** comprises a glass wafer **4** with an array of cell binding locations **8** containing protected amino groups on a hydrophobic background. **(Figure 2C).**

Fourth, the non-uniform array of cell binding locations is produced by uniformly de-protecting the amino groups in a micro-patterned chemical array produced according to the above-described methods. In one embodiment, chemical specificity can be added by chemically crosslinking specific molecules to the cell binding locations. There are a number of well known homo- or hetero-bi-functional crosslinking reagents such as ethylene glycol bis(succinimidylsuccinate) that will react with the free amino groups in the cell binding locations and crosslink to a specific molecule. Reagents and conditions for crosslinking free amino groups with other biomolecules are well known in the art, as exemplified by the following references: Grabarek and Gergely, Analyt. Biochem 185:131-135, 1990; McKenzie et al., J. Prot. Chem. 7:581-592, 1988; Brinkley, Bioconjugate Chem. 3:12-13; 1992, Fritsch et al., Bioconjugate Chem. 7:180-186, 1996; and Aplin and Hughes, 1981.

In a preferred embodiment, a non-uniform array of cell binding locations is produced in combinatorial fashion. The resulting cell binding locations are non-uniform (*i.e.*, each cell binding location or group of cell binding locations may be unique in its cell binding

selectivity). By the term combinatorial, it is meant that the cell binding locations are variably treated.

In one embodiment, the protected amino groups of the modified array of cell binding locations of step 3 are de-protected and then specific molecules with chemical crosslinking reagents are deposited in a desired pattern. The specific crosslinking agents can bind to the amino groups and further possess a cell-binding group. In this step, the type of cell binding group can be varied, from one cell binding location to another, or from one group of cell binding locations to another, to create a non-uniform design in the array.

In another embodiment, the amino groups of the chemically modified cell binding locations of step 3 are uniformly de-protected. A photo-activatable crosslinker is reacted with the de-protected amino groups. An optical mask of a desired pattern is placed over the surface of the cell binding locations and the exposed cell binding locations are illuminated with a light source. The position and number of cell binding locations that receive light is controlled by the micro-pattern of the optical mask. Suitable photo-activatable crosslinkers include aryl nitrenes, fluorinated aryl azides, benzophenones, and diazopyruvates. Reagents and conditions for optical masking and crosslinking are discussed in Prime and Whitesides, 1991; Sighvi et al., 1994, Sigal et al., 1996 and Mrksich and Whitesides, 1996. The photo-activatable crosslinker is bi-functional in that it chemically bonds to the amino group on the cell binding locations and, when exposed to light, covalently bonds to cell binding molecules, such as antibodies. Reagents and conditions for photoactivated crosslinking are discussed in Thevenin et al., Eur. J. Biochem. 206:471-477, 1992 and Goldmacher et al., Bioconjugate Chem. 3:104-107, 1992.

When a photo-activatable crosslinker is used, the glass plate is flooded with cell binding molecules to be bound to the cell binding locations. In one embodiment, cell binding molecules such as cell surface antigen-reactive antibodies, extracellular matrix proteins, (for example, fibronectin or collagen) or charged polymers (for example poly-L-lysine or poly-L-arginine) are used in concentrations ranging from about 0.1 to about 1 mM. While the cell binding molecules cover the cell binding locations, the glass plate is irradiated from the underside of the glass plate, at an angle below the critical angle of the material of the glass plate, resulting in total internal reflection of the light. (For discussion of total internal reflection fluorescence microscopy, see Thompson et al., 1993). In one embodiment, the irradiation is carried out at between ambient temperature and 37°C for 0.1 to 10 seconds with

light of wavelength between 300 nanometers (nm) to 1000 nm. In a preferred embodiment, the irradiation is conducted at ambient temperature for 1 second using light with a wavelength of between about 300 and 400 nm. Optical crosslinking limits the photo-activatable crosslinking to a short distance into the solution above the cell binding locations, and is described in Bailey et al., Nature 366:44-48, 1993; Farkas et al., Ann. Rev. Physiol. 55:785-817, 1993; Taylor et al., Soc. Opt. Instr. Eng. 2678:15-27, 1996; Thompson et al., in Mason, W.T. (ed.), "Fluorescent and Luminescent Probes for Biological Activity." San Diego: Academic Press pp. 405-419, 1993.

The photo-activatable crosslinker binds with the cell binding molecules such as antibodies and matrix proteins, only in the cell binding locations where the crosslinker was irradiated. For example, a single row of an array of cell binding locations can be irradiated to produce a single row of cell binding locations with cell binding molecules bound to the crosslinker. Following a washing of the array to eliminate any unbound cell binding molecule, a second row of cell binding locations can be bound to a second cell binding molecule by subsequent flooding of the glass wafer with the second cell binding molecule while irradiating the second row and optically masking the other rows. Unbound cell binding molecules are removed by washing the array with PBS, or any other suitable buffer. In this fashion, multiple rows of cell binding locations or groups of cell binding locations can be sequentially illuminated by sequential masking in the presence of a particular cell binding molecule. Alternatively, each cell binding location can be irradiated one by one using pinpoint exposure and optical masking. In this manner, different cell binding molecules are bound to rows of the array or to individual cell binding locations, creating a non-uniform array of cells bound to the cell binding locations of any desired pattern.

In a further embodiment for producing chemically modified arrays of cell binding locations, a chemically modified array is first produced wherein the amino groups of the cell binding locations are uniformly protected with photo-cleavable protecting groups. Rows, columns, and/or individual cell binding locations are sequentially photo-de-protected to expose the free amino groups by using an optical mask of various patterns to cover all but the cell binding locations to be de-protected. The exposed cell binding locations (*i.e.*, those not covered by the mask), are illuminated, resulting in removal of the protecting groups. The array is flooded with a bi-functional crosslinker which chemically bonds to the de-protected amino group and activates the cell binding locations. Conditions for the photode-protection

of amino groups are discussed in Padwa, A. (ed.) "Organic Photochemistry.", New York 9:225-323, 1987, Ten et al., Makromol. Chem. 190:69-82, 1989, Pillai, Synthesis 1980:1-26, 1980, Self and Thompson, Nature Medicine 2:817-820, 1996 and Senter et al., Photochem. Photobiol. 42:231-237, 1985. Next, cell binding molecules are flooded onto the modified chemical array wherein they react with the other half of the crosslinker. The array is then washed to eliminate any unbound bi-functional crosslinker and cell binding molecules. Another cell binding location or set of cell binding locations may be de-protected using another optical mask, and the array may then be flooded with a second treatment of a bi-functional crosslinker followed by a distinct cell binding molecule which bonds to this second cell binding location or set of cell binding location of de-protected amino groups. The array is washed to eliminate the second treatment of a bi-functional crosslinker and cell binding molecules. A non-uniform array of cell binding molecules may thus be produced by a repeated sequence of photo-de-protection, chemical crosslinking of specific molecules and washing under a variety of masks. Alternatively, the crosslinking reagents can be delivered to the de-protected cell binding locations together with the cell binding molecules in one step. Concentration gradients of attached cell binding molecules can be created by controlling the number of de-protected amino groups exposed using an optical mask, or by controlling the dose of irradiation for the photo-activatable crosslinkers.

The chemically modified array of cell binding locations is then used to produce a non-uniform array of cells on the cell binding locations. In one embodiment, the modified chemical array is "seeded" with cells by introducing suspended cells onto the array, allowing binding of the cells to the cell binding locations and then rinsing the wafer to remove unbound and weakly bound cells. The cells are bound only in the cell binding locations, because the specific chemical environment in the cell binding locations, in conjunction with the hydrophobic environment surrounding each of the cell binding locations, permits the selective binding of cells to the cell binding locations only. Furthermore, the modification of cell binding locations with specific cell-binding molecules permits selective binding of cells to specific cell binding locations, producing a non-uniform array of cells on the cell binding locations. In addition, the cell surface molecules that specifically bind to the cell binding locations may be either naturally present or genetically engineered by expressing "cell binding location-specific" molecules that have been fused to cellular trans-membrane molecules such that cells interact with and bind specifically to modified cell binding

locations. The creation of an array of cell binding locations with different cell recognition molecules allows one cell binding location, a group of cell binding locations, or the entire array to specifically "recognize", grow and screen cells from a mixed population of cells.

In one embodiment, cells suspended in culture medium at concentrations ranging from about 10^3 to about 10^7 cells per ml are incubated in contact with the cell binding locations for 1 to 120 minutes at temperatures ranging from ambient temperature to 37^0C. Unbound cells are then rinsed off of the cell binding locations using culture medium or a high density solution to lift the unbound cells away from the bound cells. (Channavajjala, et al., J. Cell Sci. 110:249-256, 1997). In a preferred embodiment, cells suspended in culture medium at concentrations ranging from about 10^5 to about 10^6 cells per ml are incubated in contact with the cell binding locations at 37^0C for times ranging from about 10 minutes to about 2 hours.

The density of cells attached to the cell binding locations is controlled by the cell density in the cell suspension, the time permitted for cell attachment to the chemically modified cell binding locations and/or the density of cell binding molecules in the cell binding locations. In one embodiment of the cell attachment procedure, 10^3- to 10^7 cells per ml are incubated at between ambient temperature and 37^0C for between 1 minute and 120 minutes, with cell binding locations containing between 0.1 and 100 nmoles per cm^2 of cell binding molecules. In a preferred embodiment, 10^5 and 10^6 cells per ml are incubated for 10 minutes to 2 hours at about 37^0C, with cell binding locations containing about 10 to 100 nmoles per cm^2 of cell binding molecules.

In one embodiment, the cells may be chemically fixed to the cell binding locations as described by Bell et al., J. Histochem. Cytochem 35:1375-1380, 1987; Poot et al., J. Histochem. Cytochem 44:1363-1372, 1996; Johnson, J. Elect. Micros. Tech. 2:129-138, 1985, and then used for screening at a later time with luminescently labeled molecules such as antibodies, nucleic acid hybridization probes or other ligands.

In another embodiment, the cells can be modified with luminescent indicators of cell chemical or molecular properties, seeded onto the non-uniform chemically modified array of cell binding locations and analyzed in the living state. Examples of such indicators are provided in Giuilano et al., Ann. Rev. Biophys. Biomol. Struct. 24:405-434, 1995; Harootunian et al., Mol. Biol. Cell 4:993-1002, 1993; Post et al., Mol. Biol. Cell 6:1755-1768, 1995; Gonzalez and Tsien, Biophys. J. 69:1272-1280, 1995; Swaminathan et al.,

Biophys. J. 72:1900-1907, 1997 and Chalfie et al., Science 263:802-805, 1994 . The indicators can be introduced into the cells before or after they are seeded onto the array by any one or a combination of variety of physical methods, such as, but not limited to diffusion across the cell membrane (reviewed in Haugland, Handbook of fluorescent probes and research chemicals, 6[th] ed. Molecular Probes, Inc., Eugene, 1996), mechanical perturbation of the cell membrane (McNeil et al., J. Cell Biology 98:1556-1564, 1984; Clarke and McNeil, J. Cell Science 102:533-541, 1992; Clarke et al., BioTechniques 17:1118-1125, 1994), or genetic engineering so that they are expressed in cells under prescribed conditions. (Chalfie et al., 1994). In a preferred embodiment, the cells contain luminescent reporter genes, although other types of reporter genes , including those encoding chemiluminescent proteins, are also suitable. Live cell studies permit analysis of the physiological state of the cell as reported by luminescence during its life cycle or when contacted with a drug or other reactive substance.

In another aspect of the present invention, a non-uniform cell array on the cell binding locations is provided, wherein cells are non-uniformly bound to a chemically modified array of cell binding locations on a substrate. The cell array is non-uniform because the underlying non-uniform chemically modified array of cell binding locations provides a variety of cell binding sites of different specificity. Any cell type can be arrayed, providing that a molecule capable of specifically binding that cell type is present in the chemically modified array of cell binding locations. Preferred cell types for the non-uniform cell array on the cell binding locations include lymphocytes, cancer cells, neurons, fungi, bacteria and other prokaryotic and eukaryotic organisms. For example, **Figure 3A** shows a non-uniform cell array on the cell binding locations containing fibroblastic cells grown on a surface patterned substrate and labeled with two fluorescent probes (rhodamine to stain actin and Hoechst to stain nuclei), while **Figure 3B** shows a non-uniform cell array on the cell binding locations containing fibroblastic cell growth (L929 and 3T3 cells) in spotted patterns, labeled with two fluorescent probes and visualized at different magnifications.

Examples of cell-binding molecules that can be used in the non-uniform cell array on the cell binding locations include, but are not limited to antibodies, lectins and extracellular matrix proteins. Alternatively, genetically engineered cells that express specific cell surface markers can selectively bind directly to the modified cell binding locations. The non-uniform cell array on the cell binding locations may comprise either fixed or living cells. In a

preferred embodiment, the non-uniform cell array on the cell binding locations comprises living cells such as, but not limited to, cells "labeled" with luminescent indicators of cell chemical or molecular properties.

In another aspect of the present invention, a method for analyzing cells is provided, comprising preparing a non-uniform cell array on the cell binding locations wherein the cells contain at least one luminescent reporter molecule, contacting the non-uniform cell array on the cell binding locations to a fluid delivery system to enable reagent delivery to the cells, conducting high-throughput screening by acquiring luminescence image of the entire non-uniform cell array at low magnification to detect luminescence signals from all cell binding locations at once to identify those that exhibit a response. This is followed by high-content detection within the responding cell binding locations using a set of luminescent reagents with different physiological and spectral properties, scanning the selected cell binding locations to obtain luminescence signals from the luminescent reporter molecules in the cells, converting the luminescence signals into digital data and utilizing the digital data to determine the distribution, environment or activity of the luminescent reporter molecules within the cells.

Preferred embodiments of the non-uniform cell array on the cell binding locations are disclosed above. In a preferred embodiment of the fluid delivery system, a chamber, mates with the substrate containing the non-uniform cell array. The chamber is preferably made of glass, plastic or silicon, but any other material that can provide a chamber is suitable. One embodiment of the chamber **12** shown in **Figure 4** has an array of etched domains **13** matching the cell binding locations **8** on the substrate **4**. In addition, input channels **14** are etched to supply fluid to the etched domains **13**. A series of "output" channels **16**, to remove excess fluid from the etched domains **13**, can also be connected to the cell binding locations. The chamber **12** and substrate **10** together constitute a cassette **18**. While this embodiment utilizes etched domains, any other type of depression **13** formed at the fluidic location **1 can** also be utilized in this embodiment. Alternatively, the fluidic location may be flat and the cell binding locations **8** may comprise depressions that match the fluidic locations **1**. In another alternative, both the cell binding site **8** and the fluidic location **1** are flat, and a volume space for the well is created by the use of a spacer support **20** between the substrate **4** and the chamber **12.**

The chamber **12** is thus used for delivery of fluid to the cells arrayed on the cell binding locations **10**. The fluid can include, but is not limited to a solution of a particular drug, protein, ligand, or other substance which binds with surface expressed moieties of cells or that are taken up by the cells. The fluid to interact with the cells arrayed on the cell binding locations **10** can also include liposomes encapsulating a drug. In one embodiment, such a liposome is formed from a photochromic material, which releases the drug upon exposure to light, such as photoresponsive synthetic polymers. (Reviewed in Willner and Rubin, Chem. Int. Ed. Engl. 35:367-385, 1996). The drug can be released from the liposomes in all channels **14** simultaneously, or individual channels or separate rows of channels may be illuminated to release the drug sequentially. Such controlled release of the drug may be used in kinetic studies and live cell studies. Control of fluid delivery can be accomplished by a combination of micro-valves and micro-pumps that are well known in the capillary action art. (United States Patent No. 5,567,294; United States Patent No. 5,527,673; United States Patent No. 5,585,069, all herein incorporated by reference.)

Another embodiment of the chamber **12** shown in **Figure 5** has an array of input channels **14** matching the chamber's etched domains **13** which are slightly larger in diameter than the cell binding locations **8** on the substrate **4**, so that the cell binding sites are immersed into the etched domains **13** of the chamber **12**. Spacer supports **20** are placed between the chamber **12** and the cells arrayed on the cell binding locations **10** along the sides of contact. The substrate **4** and the chamber **12** can be sealed together using an elastomer or other sticky coating on the raised region of the chamber. Each etched domain **13** of the chamber **12** can be individually or uniformly filled with a medium that supports the growth and/or health of the cells arrayed on the cell binding locations **10**. In a further embodiment **(Figure 6)**, the chamber contains no input channels, for treating all the cells arrayed on the cell binding locations **10** with the same solution.

Delivery of drugs or other substances is accomplished by use of various modifications of the chamber as follows. A solution of the drug to be tested for interaction with cells of the array can be loaded from a 96 well microtiter plate into an array of micro-capillary tubes **24**. **(Figure 7)**. The array of micro-capillary tubes **24** corresponds one-to-one with the input channels **14** of the chamber **12**, allowing solution to flow or be pumped out of the micro-capillary tubes **24** into the channels **14**. The cassette **18** is inverted so that the cell binding locations **8** become submerged in the etched domain **13** filled with the fluid **(Figure 7B)**.

Once the interaction between the fluid and cells occurs, luminescence signals emanating from the cells arrayed on the cell binding locations **10** can be measured directly or, alternatively, the substrate **4** can be lifted off the chamber for post processing, fixation, and labeling. The placement and removal of the array of cells may be accomplished via robotics and/or hydraulic mechanisms. (Schroeder and Neagle, 1996)

In one embodiment of the chamber **12** shown in **Figure 7,** the channels and matching etched domains **13** are etched into the chamber chemically (Prime and Whitesides, 1991; Lopez et al., 1993; Mrksich and Whitesides, 1996). The etched domains **13** are larger in diameter than the cell binding locations **8.** This permits the chamber **12** to be contact sealed to the substrate **4,** leaving space for the cells and a small volume of fluid. Input channels **14** are etched into each row of etched domains **13** of the chamber **12.** Each input channel **14** extends from two opposing edges of the chamber **12** and is open at each edge. The etched domains **13** of a single row are in fluid communication with the input channels **14** by placing a micro-capillary tube **24** containing a solution into contact with the edge of the chamber **12.** Each row of connected input channels **14** can be filled simultaneously or sequentially. During filling of the input channels **14** by valves and pumps or capillary action, each of the channels of the chamber **12** fills and the drug passes to fill each etched domain **13** in the row of etched domains **13** connected by the input channel **14.**

In a further embodiment of the chamber **12,** raised reservoirs **28** and input channels **14** can be placed onto the surface of the chamber **12** as shown in **Figure 8b.** In a preferred embodiment, the raised reservoirs **28** and input channels **14** can be made from polytetrafluoroethylene or elastomeric material, but they can be made from any other sticky material that permits attachment to the substrate **4,** such as poly(dimethylsiloxane), manufacture by Dow Corning under the trade name SYLGARD 184™. The effect is the same as with a chamber having etched channels and channels and its uses are similar.

In another embodiment of the chamber shown in **Figure 8A,** a first channel **30** extends from one edge of the chamber **12** to a first etched domain **13** or raised reservoirs **28** and channels. A second channel **32** extends from the opposing edge to a second etched domain adjacent the first etched domain. The first **30** and second **32** channels are not in fluid communication with each other yet are in the same row of input channels **14** or raised reservoirs **28.**

In another embodiment, as shown in **Figures 9 and 10**, the chamber **12** may have an input channel **14** extending from each etched domain **13** or raised reservoir **28** to the edge of the chamber. The channels **14** can all originate from one edge of the chamber **12 (Figure 9)**, or from both edges (**Figure 10**). The input channels **14** can also be split to both sides of the etched domains **13** to minimize the space occupied by the input channels **14**. Separate fluidic channels allow for performance of kinetic studies where one row at a time or one depression at a time is charged with the drug.

In a further embodiment depicted in **Figure 11**, each etched domain **13** is in fluid communication with a corresponding input channel **14** having a plug **36** between the end of the channel **14** and the etched domain **13**, which prevents the injected solution from flowing into the etched domain **13** until the desired time. Solutions may be preloaded into the input channels **14** for use at a later time. A plug **36** likewise can be disposed between a terminal etched domain **13** in a set of connected etched domains **13** in fluid communication with an input channel **14**. Upon release of the plug **36**, the substance flows through and fills all the etched domains **13** which are in fluid communication with the input channel **14**.

In one embodiment, the plugs **36** are formed of a hydrophobic polymer, such as, but not limited to proteins, carbohydrates or lipids that have been crosslinked with photo-cleavable crosslinkers that, upon irradiation, becomes hydrophilic and passes along with the drug into the depression. Alternatively, the plug **36** may be formed of a crosslinked polymer, such as proteins, carbohydrates or lipids that have been crosslinked with photo-cleavable crosslinkers that, when irradiated, decomposes and passes into the etched domain **13** along with the solution.

The cassette **18,** which comprises the substrate **4** and the chamber **12,** is inserted into a luminescence reader instrument. The luminescence reader instrument is an optical-mechanical device that handles the cassette, controls the environment (*e.g.*, the temperature, which is important for live cells), controls delivery of solutions to wells, and analyzes the luminescence emitted from the array of cells, either one well at a time or the whole array simultaneously. In a preferred embodiment **(Figure 12)**, the luminescence reader instrument **44** comprises an integrated circuit inspection station using a fluorescence microscope as the reader and microrobotics to manipulate the cassettes. The reader of the present invention can comprise any optical system designed to image a luminescent specimen onto a detector. A storage compartment **48** holds the cassettes **18**, from where they are retrieved by a robotic

arm **50** that is controlled by computer **56.** The robotic arm **50** inserts the cassette **18** into the luminescence reader instrument **44.** The cassette **18** is removed from the luminescence reader instrument **44** by another robotic arm **52,** which places the cassette **18** into a second storage compartment **54.**

The luminescence reader instrument **44** is an optical-mechanical device designed as a modification of light optical-based, integrated circuit inspection stations used to "screen" integrated circuit "chips" for defects. Systems integrating environmental control, micro-robotics and optical readers are produced by companies such as Carl Zeiss [Jena, GmbH]. In addition to facilitating robotic handling, fluid delivery, and fast and precise scanning, two reading modes, high content and high throughput are supported. High-content readout is essentially the same as that performed by the ArrayScan reader (U.S. Application S/N 08/810983). In the high content mode, each location on the micro-array of multiple cell types is imaged at magnifications of 5-40X or more, recording a sufficient number of fields to achieve the desired statistical resolution of the measurement(s).

In the high throughput mode, the luminescence reader instrument **44** images the micro-array of multiple cell types at a much lower magnification of 0.2X to 1.0X magnification, providing decreased resolution, but allowing all the cell binding locations on the substrate to be recorded with a single image. In one embodiment, a 20mm x 30mm micro-array of multiple cell types imaged at 0.5 X magnification would fill a 1000 x 1500 array of 10um pixels, yielding 20um/pixel resolution, insufficient to define intracellular luminescence distributions, but sufficient to record an average response in a single well, and to count the numbers of a particular cell subtype in a well. Since typical integration times are on the order of seconds, the high throughput mode of reading technology, coupled with automated loading and handling, allows for the screening hundreds of compounds a minute.

In one embodiment shown in **Figure 13,** the luminescence reader instrument comprises an optical-mechanical design that is either an upright or inverted fluorescence microscope **44,** which comprises a computer-controlled x,y,z-stage **64,** a computer-controlled rotating nosepiece **68** holding a low magnification objective **70** (*e.g.*, 0.5 x) and one or more higher magnification objectives **72,** a white light source lamp **74** with excitation filter wheel **76,** a dichroic filter system **78** with emission filters **80,** and a detector **82** (*e.g.*, cooled charge-coupled device). For the high throughput mode, the low magnification objective **70** is moved into place and one or more luminescence images of the entire cell array is recorded. Wells

that exhibit some selected luminescence response are identified and further analyzed via high content screening, wherein the nosepiece **68** is rotated to select a higher magnification objective **72** and the x,y,z-stage **64** is adjusted to center the "selected" well for both cellular and subcellular high content screening, as described in U.S. Application S/N 08/810983.

In an alternate embodiment, the luminescence reader instrument **44** can utilize a scanned laser beam in either confocal or standard illumination mode. Spectral selection is based on multiple laser lines or a group of separate laser diodes, as manufactured by Carl Zeiss (Jena, GmbH, Germany) or as discussed in Denk, et al. (Science 248:73, 1990).

Another embodiment of the high throughput screening mode involves the use of a low-resolution system consisting of an array (1 x 8, 1 x 12, etc.) of luminescence exciters and luminescence emission detectors that scans subsets of the wells on a non-uniform micro-patterned array of cells. In a preferred embodiment, this system consists of bundled optical fibers, but any system that directs luminescence excitation light and collects luminescence emission light from the same well will suffice. Scanning the entire micro-array of multiple cell types with this system yields the total luminescence from each well, both from cells and the solution they are bathed in. This embodiment allows for the collection of luminescence signals from cell-free systems, so-called "homogeneous" assays.

Figure 14A shows a method, in the form of a flow chart, for analyzing a micro-array of multiple cell types in both the high throughput and high content modes using the luminescence reader instrument, which first uses high throughput detection to measure a response from the entire array "A". **(Figure 14B)**. Any well that responds above a preset threshold is considered a hit and the cells in that well are measured via high content screening. **(Figure 14C)**. The high content mode ("B") may or may not measure the same cell parameter measured during the high throughput mode ("A").

In another aspect of the invention, a cell screening system is disclosed, wherein the term "screening system" comprises the integration of a luminescence reader instrument, a cassette that can be inserted into the luminescent reader instrument comprising a micro-array of multiple cell types wherein the cells contain at least one luminescent reporter molecule and a chamber associated with the non-uniform micro-patterned array of cells, a digital detector for receiving data from the luminescence reader instrument, and a computer means for receiving and processing digital data from the digital detector.

Preferred embodiments of the luminescence reader instrument, and the cassette comprising the micro-array of multiple cell types and the chamber are disclosed above. A preferred embodiment of the digital detector is disclosed in U.S. Application S/N 08/810983, and comprises a high resolution digital camera that acquires luminescence data from the luminescence reader instrument and converts it to digital data. In a preferred embodiment, the computer means comprises a digital cable that transports the digital signals from the digital detector to the computer, a display for user interaction and display of assay results, a means for processing assay results, and a digital storage media for data storage and archiving, as described in U.S. Application S/N 08/810983.

In a preferred embodiment, the cell screening system of the present invention comprises integration of the preferred embodiments of the elements disclosed above (**Figure 15**). The array of multiple cell types **10** comprises cells bound to chemically modified cell binding locations **8** on a substrate **4**. The chamber **12** serves as a microfluidic delivery system for the addition of compounds to the cells **10** on the substrate **4**, and the combination of the two comprises the cassette **18**. The cassette **18** is placed in a luminescence reader instrument **44**. Digital data are processed as described above and in U.S. Application S/N 08/810983, hereby incorporated by reference in its entirety. The data can be displayed on a computer screen **86** and made part of a bioinformatics database **90**, as described in U.S. Application S/N 08/810983. This database **90** permits storage and retrieval of data obtained through the methods of the invention, and also permits acquisition and storage of data relating to previous experiments with the cells. An example of the computer display screen is shown in **Figure 16.**

Example 1. Coupling of antibodies to micro-array of multiple cell types for the attachment of specific lymphoid cells

1. The cell line used was a mouse B cell lymphoma line (A20) that does not express IgM on its surface. A micro-array of multiple cell types was prepared for derivatization by being immersed overnight in 20% sulfuric acid, washed 2-3 times in excess distilled water, rinsed in 0.1M sodium hydroxide and blotted dry. The micro-array of multiple cell types was either used immediately or placed in a clean glass beaker and covered with parafilm for future use.

2. The micro-array of multiple cell types was placed in a 60mm petri dish, and 3-Aminopropyltrimethoxysilane was layered onto the micro-array of multiple cell types ensuring complete coverage without running over the edges (approximately 0.2ml for a 22 x 22 mm non-uniform micro-patterned array of cells, and approximately 0.5ml for a 22 x 40 mm non-uniform micro-patterned array of cells). After 4 minutes at room temperature, the micro-array of multiple cell types was washed in deionized water and excess water was removed by blotting.
3. The micro-array of multiple cell types was placed in clean 60mm petri dishes and incubated with glutaraldehyde (2.5% in PBS, approximately 2.5ml) for 30 minutes at room temperature, followed by three PBS washes. Excess PBS was removed by blotting.
4. Cell nuclei in the micro-array of multiple cell types were labeled with a luminescent Hoechst dye during the blocking step. The appropriate number of lymphoid cells (see below) in C-DMEM were transferred to a 15 ml conical tube, and Hoechst dye was added to a final concentration of 10 µg/ml. Cells were incubated for 10-20 minutes at 37°C in 5% CO_2, and then pelleted by centrifugation at 1000 x *g* for 7 minutes at room temperature. The supernatant containing unbound Hoechst dye was removed and fresh media (C-DMEM) was added to resuspend the cells as follows: approximately 1.25 –1.5 x 10^5 cells in 0.2 ml per 22 x 22 mm non-uniform micro-patterned array of cells, and approximately 2.5 x 10^5 cells in 0.75ml for the 22 x 40 mm non-uniform micro-patterned array of cells.
5. The micro-array of multiple cell types was washed briefly in PBS and transferred to a clean, dry 60 mm petri dish, without touching the sides of the dish. Cells were carefully pipeted onto the top of the micro-array of multiple cell types at the density noted above. Dishes were incubated at 37°C in 5% CO_2 for 1 hour. Unbound cells were then removed by repeated PBS washings.
6. Antibody solutions (Goat Anti-Mouse IgM or Goat Anti-Mouse Whole Serum) were spotted onto parafilm (50 µl for 22 x 22 mm non-uniform micro-patterned array of cells, 100 µl for a 22 x 40 mm non-uniform micro-patterned array of cells). The micro-array of multiple cell types was inverted onto the spots, so that the antiserum covered the entire surface of the treated micro-array of multiple cell types without trapping air bubbles. The micro-array of multiple cell types was incubated with the antibody solution for 1 hour at room temperature.

7. The micro-array of multiple cell types was carefully lifted from the parafilm, placed in a clean 60 mm petri dish, and washed three times with PBS. Unreacted sites are then blocked by the addition of 2.5 ml of 10% serum (calf or fetal calf serum in DMEM or Hank's Balanced Salt Solution) for 1 hour at room temperature.
8. Both cell lines should bind to the anti-mouse whole serum, but only the X16s should bind to the anti-mouse IgM. The binding of specific lymphoid cell strains to the chemically modified surface is shown in **Figure 17**. The mouse lymphoid A20 cell line, lacking surface IgM molecules but displaying IgG molecules, bound much more strongly to the surface modified with whole goat anti-mouse serum **(Figure 17C)** than to the surface modified with goat anti-mouse IgM **(Figure 17B)** or an uncoated slide **(Figure 17A)**.

Example 2. High-Content and High Throughput Screen.

The insulin-dependent stimulation of glucose uptake into cells such as adipocytes and myocytes requires a complex orchestration of cytoplasmic processes that result in the translocation of GLUT4 glucose transporters from an intracellular compartment to the plasma membrane. A number of molecular events are triggered by insulin binding to its receptor, including direct signal transduction events and indirect processes such as the cytoskeletal reorganizations required for the translocation process. Because the actin-cytoskeleton plays an important role in cytoplasmic organization, intracellular signaling ions and molecules that regulate this living gel can also be considered as intermediates of GLUT4 translocation.

A two level screen for insulin mimetics is implemented as follows. Cells carrying a stable chimera of GLUT4 with a Blue Fluorescent Protein (BFP) are arranged on the micro-array of multiple cell types arrays, and then loaded with the acetoxymethylester form of Fluo-3, a calcium indicator (green fluorescence). The array of locations are then simultaneously treated with an array of compounds using the microfluidic delivery system, and a short sequence of Fluo-3 images of the whole micro-array of multiple cell types are analyzed for wells exhibiting a calcium response in the high throughput mode. The wells containing compounds that induced a response, are then analyzed on a cell by cell basis for evidence of GLUT4 translocation to the plasma membrane (*i.e.*, the high-content mode) using blue fluorescence detected in time and space.

Figure 18 depicts the sequential images of the whole micro-array of multiple cell types in the high throughput mode (**Figure 18A**) and the high content mode (**Figure 18B**). **Figure 19** shows the cell data from the high content mode.

Improved Cassettes and Enhanced Well Density

In another aspect, the present invention provides devices and methods for maximizing the cell-plated area and the number of wells that can be imaged in a sub-array, while still obtaining adequate pixel resolution in the image. This result has been achieved through the use of fluidic architectures that minimize the distance and area between wells, and thus maximize well density.

In one embodiment of this aspect is provided a cassette for cell screening comprising a substrate having a surface, wherein the surface contains a plurality of cell binding locations; a fluid delivery system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the substrate, wherein the multi-level chamber comprises

i. a crossed array of microfluidic input channels and output channels, wherein each well is in fluid connection with one or more input channels and one or more output channels;

ii. a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels;

iii. one or more input manifolds in fluid connection with the microfluidic input channels;

iv. one or more output manifolds in fluid connection with the microfluidic output channels;

v. at least one source receptacle in fluid connection with the one or more input manifolds; and

vi. at least one waste receptacle in fluid connection with one or more output manifolds; and

a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location.

In preferred embodiments, the cassettes further comprise a pump to control fluid flow within the microfluidic device; a substrate temperature controller and/or a controller to regulate oxygen and carbon dioxide partial pressures within the device.

The substrate of the present invention is chemically modified so that cells selectively adhere to a cell growth substrate and are contained within small regions, referred to as "cell binding locations", which are sub-millimeter to a few millimeters in size. The combination of a cell binding location on the substrate and a fluidic location on the chamber defines a space referred to as a "well". The substrate may be predominantly flat and the cell binding locations may be matched with fluidic location depressions on a substrate cover, so that when the two parts are assembled there is some depth in the area of the wells so formed. Conversely, the substrate cover fluidic location may be flat and the substrate may contain depressions at the cell binding location, so that when the two parts are assembled there is some depth in the area of the wells so formed. Fluids and test compounds are supplied to the wells via a chamber, which combines with the substrate to form a cassette. In a preferred embodiment, the chamber acts as the substrate cover and contains fluidic locations in fluid connection with the microfluidic channels of the device, wherein the fluidic locations comprise depressions that match the cell binding locations on the substrate, such that the volume of each well is etched into the chamber.

On the surface of the substrate, there are sealing regions that lie between and around the cell binding locations, and there are corresponding sealing regions on the bottom face of the chamber that lie between and around the fluidic locations. When the substrate and the chamber are joined, the sealing regions of both components meet to form a seal that prevents the flow of fluid across the sealing regions between the wells thereby formed. These sealing regions of both components may be physically and/or chemically modified to enhance sealing. In a first example, a hydrophobic silane coating of octadecyltrichlorosilane is coated on the sealing regions. In a second example, light-curable adhesives and cyanoacrylates that are USP Class IV-compliant such as are available from Dymax Corporation are amenable to use with biomedical devices made from ceramic, glass, plastic, or metal. In a third example, a biocompatible tape material with precision cut-outs that match the wells of the array is available from Avery Dennison Specialty Tape Division. Similarly, biomedical acrylic adhesives can be transferred from tapes to surfaces, such as are available from Tyco Corporation, for applying an adhesive coating precisely to these sealing regions. In a fourth

example, a light-curable silicone elastomer can be used for bonding and sealing between the sealing regions of the substrate and the chamber, such as are available from Master Bond, Inc.

The microfluidic cassette of the present invention has advantages over prior art cell array microfluidic devices used in high content screening systems including, but not limited to:

1) faster imaging of a statistically-relevant set of cells (based on imaging a set of cell binding locations (i.e.: "sub-arrays") simultaneously; See **Fig. 20**)
2) more wells per plate and less physical space occupied per plate (i.e.: higher well density),
3) more efficient use of valuable test compounds, and
4) more efficient use of cells compared to microplates in which the entire well area is not imaged.

As discussed *supra*, prior microwell plate fluid exchange for each well can only be achieved by means of a pipette being inserted into the well and either ejecting or aspirating fluid to or from the well, respectively. Automated fluid handling in prior microwell plates is acheived by using robotic pipettors that insert a pipette into each well for each fluid transfer step in a procedure. This pipette is typically required also to move to or from another microplate well for the source or destination of the fluid that is exchanged with the microwell plate of interest.

Integrated fluidics is advantageous for arrays with sub-millimeter inter-well distances because it is unwieldy, if not impossible, to pipette fluids with an acceptable degree of spatial resolution and accuracy. An acceptable level of accuracy requires that the measurement error should not exceed 2%. Thus, for a 100 microliter (μl) volume in the well of a 96 well plate, a measurement error of 2 μl is acceptable. For a 100 nano-liter (nl) volume, a 2 nl measurement error would be required. This level of accuracy is not reliably available with prior automatic pipettors across a spectrum of compositions and viscosities.

The minimization of the inter-well distances enables the fast parallel reading of sub-arrays of wells. If the integrated fluidics is too bulky (i.e.: does not allow close-spacing of wells), then such fast parallel reading capability is lost. In a preferred embodiment of the present invention, a crossed channel architecture is utilized, allowing for fewer channels, valves, and pumps, and thereby further reducing the space taken by channels on the cassette

of the invention. In a most preferred embodiment, the channels are placed in levels above that of the wells, permitting the closest possible inter-well spacing. In a further preferred embodiment, the use of porous medium as a drain 'pump' allows for simpler design and fabrication of the system. According to the invention, the channels may be of any size that permits the fluidic architecture herein defined. In a preferred embodiment, they range in size from between about 0.025 mm to about 0.5 mm in width for square cross-section channels, or in diameter for circular cross-section channels.

The fluidic architecture of the instant microfluidic cassette enables the controlling elements (including, but not limited to pumps, manifolds, controlled-pressure vessels, and/or valves) to be located outside the matrix of the cell array. Figure 40 shows one embodiment of the pumping and valving schemes of this invention as it would appear with all the pumps and valves on-board the cassette. All variations of the pumping and valving schemes shown in various Figures (and others that would be apparent to those skilled in the art) may be implemented in this way, either with on-board active devices outside the matrix of the wells, with off-board active devices, or with some devices on-board and some devices off-board. As a result, the controlling elements can be either within the cassette (on-board) or external to the cassette (off-board). In either case, there are no active components within the matrix of the array of wells that might limit the close-spacing of channels and wells. Because this cassette is particularly designed for the culture and analysis of living cells, the fluidic architecture and all of its sub-components and functional parts are compatible with, support, and enable the culture of living cells. The particular aspects of these sub-components and functional parts that are designed for live cell culture are identified below.

In a preferred embodiment, the cassette of the present invention comprises an off-board pump to provide pressure-driven flow that also controls which wells are addressed by the flow. This design feature ensures that the pumping method, unlike many prior art methods, is not ineffective or harmful when used with cell culture medium that is aqueous, polar and contains proteins and salts. For example, electrohydrodynamic pumping is ineffective with polar solvents (Marc Madou, Fundamentals of Microfabrication, CRC Press, Boca Raton, 1997, p. 433). Electro-osmosis is typically accompanied by some degree of electrophoretic separation of charged medium components, such as proteins. Also, electro-osmosis typically requires the use of electric field strengths within the range of 100 V/cm to 1000 V/cm, which may affect the physiology of the living cells in the device. The fluid

control system of the present device does not suffer from the disadvantages of electrically-induced methods when using biological fluids. .

In another embodiment, the pump is on-board and may utilize electrically-driven flow, but the electric fields are limited to regions external to the matrix of the array of wells, and no electric field gradients are applied across any of the wells in which live cells are present. This embodiment is shown in **Figure 41**, where two electrodes are used to apply electric potential differences along segments of fluidic channels corresponding to different on-board source reservoirs (730). Each channel segment with its electrode pair form an electrokinetic pump (740). These source reservoirs (730) may be supplied with media and/or compounds via fill ports (700) in the cassette surface. Valves (720) between the reservoirs and the pumps are closed whenever the corresponding electrokinetic pump is off, and are open whenever the corresponding electrokinetic pump is on, to maintain back pressure and to allow intake of air or fluid, respectively. Control of the voltage applied to electrode 1 (760) is used to induce electrokinetic flow of the fluid in the corresponding channel segment. Electrode 2 (780) (nearest to the matrix of wells) is kept at ground potential, as is the matrix of the wells, to ensure that minimal or no potential difference and/or electric field is developed in the vicinity of the cells, because electric fields are known to affect cell physiology. Alternatively, the electrokinetic pump may be located down stream from the array to serve as a negative pressure pump. In this embodiment, electrophoresis of the media by the pumping action, if any, would only affect the media after it has been already used by the cells and is on its way to the waste reservoir.

In all embodiments, the use of on-board or off-board pumps that are external to the matrix of wells to control fluid flow eliminates the need for active valves within the matrix of wells, and thus eliminates the problem of allocating space within the matrix that might prevent the close spacing of the wells.

The control of the fluid pathway by means of pressure control also enables the optional use of various diffusion control means that are compatible and effective with cell culture medium that is aqueous, polar and contains proteins and salts. These means also require minimal space on the device, and thus do not prevent the close-spacing of wells.

U.S. Patent No. 5,603,351 describes a microfluidic device that includes crossed channels, but the channel network is not defined to allow two or more reagents to be combined in a reaction well, but rather to allow reagents to be fed to a well in a serial fashion.

The purpose of the present **cassette** is not to expose two or more different fluids to each other, but to expose the living cells cultured on the well bottoms in serial fashion to two or more different fluids.

The fluidic channels of the cassette of the present invention provide transport of fluid and compounds from source receptacles to each well and from each well to one or more waste receptacles. Each well could be provided with a separate input and output channel (**Fig. 21**), yielding $2n^2$ channels (100, 120) for an $n \times n$ array (040). To control the flow of m liquid or vapor mixtures to an $n \times n$ array, $m + n^2$ valves or pumps are required (**Fig. 22**). However, for a large array, the number of channels, pumps, and valves for this type of architecture becomes unwieldy. A pump that has variable or switchable pressure (e.g., a syringe pump) is functionally equivalent to a pressurized reservoir with a valve. Therefore, in the following discussions of pumps, reservoirs, and valves, it should be understood that these components may be substituted without altering the spirit of the invention.

Crossed-channel multilevel architecture

A preferred embodiment of architectures of the cassette consisting of a crossed, nonintersecting array of input (100) and output (120) channels and utilizing multiple levels is disclosed. Specific embodiments within this class of architectures are given in **Figs. 23-26** and in **Figs. 33** and **34**.

Because architectures in this class require fewer independent channels per well compared to the class of architectures with separate input and output channels for each well, these crossed-channel architectures also minimize the lateral space (140) that is required between wells to accommodate the pathways for the channels.

The crossed-channel, multilevel architectures require only $2n$ channels to address n^2 wells. In a preferred embodiment, the row and column channels themselves are formed in two distinct layers so that they do not intersect. The row channels lie above the column channels, or vice versa. These two layers lie either in the same plane of the well layer (**Figures 23 and 24**), or lie in a plane above the well layer (**Figs. 25-26** and **33-34**). In other embodiments, structures with more than two planes are contemplated.

In a most preferred embodiment, the channels are placed above the plane of the wells, so that the minimum inter-well distance (140) is constrained only by the optical and physical requirements for a certain wall thickness separating each well. The advantage of placing the channels in levels above the wells is shown by the following examples. Wells that are 0.4 mm x 0.4 mm can be placed on a 0.5 mm pitch grid with allowance for a 0.1 mm wall between wells, yielding a 5 mm x 5 mm area for a 10 x 10 well array. Row and column channels of 0.2 mm width can easily be formed in upper levels of the device with ample distance between the channels. By contrast, if 0.2 mm channels with 0.1 mm walls are required within the plane of the wells (and row and column channel layers are formed in two separate planes within the plane of the wells), the well pitch increases to 0.8 mm. This yields an 8 mm x 8 mm area for a 10 x 10 well array. In this example, the placement of the channels between the wells increases the area required for the array by a factor of 2.6. For different channel architectures with separate input and output channels for each well, the additional space required between wells is much greater.

Either for channels above or within the level of the wells, horizontal (160) or vertical (180) connecting channels or vias **are** formed from each well ***(j,k)*** to its corresponding row channel ***j*** and column channel ***k***. Fabrication of such structures from glass, semiconductor, or plastic materials is well known in the art. In one such method, silicon dioxide (SiO_2) is lithographically formed within silicon nitride (Si_3N_4) layers using a multistep layer-by-layer process. (Turner and Craighead, Proc. SPIE 3528:114-117 (1998)) Exposure of the structure to a wet etchant removes the SiO_2 to create the channel network within the Si_3N_4, which is not etched.

Control of flow in the crossed-channel architecture

In a crossed array of rows ***(A, B, C, etc.)*** and columns ***(1, 2, 3, etc.)*** the flow of fluid to and from well ***(j,k)*** is produced when, for example, positive pressure is applied to row ***j*** and the valve of output channel ***k*** is opened, where ***j*** and ***k*** refer to, e.g., row ***D*** and column *7*, respectively. Flow to all wells of an entire row ***j*** is produced when, for example, positive pressure is applied to row ***j*** and the valves of all output channels ***(1, 2, 3, etc.)*** are opened. A similar procedure would yield flow to all wells of a column.

The pressures that are discussed here are actually pressure differences relative to ambient atmospheric pressure, otherwise known as "gauge" pressure. Thus, the value of the

positive pressure is the amount by which an applied pressure is greater than atmospheric pressure; the value of the negative pressure is the amount by which an applied pressure is less than atmospheric pressure. These pressures are referred to as positive and negative gauge pressure, respectively.

The term fluid pathway is used herein indicates the particular set of wells {(j,k), (j,n), (m,k), (m,n) ... etc.} that is subjected to fluid flow by the enabling of the flow control devices of rows j and m, etc., and columns k and n, etc. Note that enabling the flow control devices of two rows and two columns yields a fluid pathway through four wells. This is defined herein as a single fluid pathway involving four wells, rather than four pathways involving single wells.

Reduction in the number of active valves or pumps

Various combinations of actively-controlled flow control devices (pumps or pressurized reservoirs with valves) are used to produce flow through a desired well (010) or fluid pathway. For example, *m* compounds can be multiplexed to a single well by using *m* positive pressure source reservoirs or pumps (200), a valve-less manifold (240) and valve-free waste reservoir at atmospheric pressure (300)(**Fig. 27**). This valve-less manifold (240) can be replaced by a valved manifold [e.g., (260) of **Fig. 28**] to better control the diffusion of fluids between the *m* sources. Alternatively, the same functionality can be achieved by using a negative pressure waste reservoir or pump (320) with *m* valves (260) connected to *m* source reservoirs at atmospheric pressure (220)(**Fig. 28**). The use of source reservoirs at atmospheric pressure allows convenient bubbling of gases through the source reservoirs to establish and maintain desired levels of dissolved CO_2 and O_2 in the media.

In another embodiment, a waste reservoir filled with a porous medium serves as a capillary action pump (340) and provides negative pressure (**Fig. 28**). The thermodynamics of wetting creates a pressure across a liquid-vapor interface bounded by a solid-liquid-vapor contact line. This pressure of capillary action can be used to draw a column of liquid from any microchannel pathway into the high surface area porous medium. A series of such negative pressure reservoirs can be linked to the array via a multiplexing valved manifold for control of flow, or one single reservoir can be closely coupled to the output channels of all wells simultaneously. A number of porous media are known in the art, including, but not limited to silica gel technology, porous ceramic materials such as zeolite, a pad of hydrophilic

(synthetic or natural) fibers, and partially hydrated or lyophilized hydrogels such as alginates, poly(vinyl) alcohol gels, agarose, sugar polyacrylate gels, and polyacrylamide gels. In a preferred embodiment, the porous medium is based on silica gel technology. A thin membrane or filtering sheet may be used to partition the porous media from the microchannel array. This method can provide pressure for pumping until the internal surface area of the porous medium is largely wetted by the liquid. By suitable choice of porous medium and design of this method, adequately large amounts of liquid can be pumped to conduct a chosen measurement with the array system of the present invention.

In another embodiment, the array of m positive pressure source reservoirs or pumps (200) shown in **Fig. 27** is multiplexed to an $n \times n$ crossed channel array of wells (040) by means of $1 \times n$ input (400) and $n \times 1$ output (410) valve manifolds (**Fig. 29**). Recall that for architectures with separate input and output channels for each well (**Fig. 22**), $m + n^2$ valves or pumps are required in this situation. Again, the valve-less manifold (240) can be replaced by a valved manifold [e.g., (260) of **Fig. 28**] to better control the diffusion of fluids between the m sources. Flow through well (j,k) is effected when input valve j and output valve k are opened. Alternatively, the same functionality can be achieved with m reservoirs at atmospheric pressure (220) and a negative pressure waste reservoir (either 320 or 340) as in **Fig. 28** by means of $1 \times n$ input (400) and $n \times 1$ output valve (410) manifolds (Fig. 30).

Rinse channels

In a preferred embodiment, an $n \times n$ microwell array is augmented by an additional column channel k^* or set of column channels(s) $\{k^*\}$ that allow passing of a segment of liquid or vapor from a given row j to a waste reservoir without going through the wells $\{(j,k)\}$ or the column channels $\{k\}$. These additional column channels $\{k^*\}$ are referred to as <u>rinse channels</u> (130), because they enable the rinsing of the row channels without requiring flow through the wells.

In general, a rinse channel k^* (130) can be located adjacent and parallel to column channel k **(016)** but be linked by a <u>via</u> to <u>row channel</u> j **(014)** at the point were they cross [and <u>not</u> be linked directly to the corresponding <u>well</u> (j,k)]. In one embodiment, <u>every</u> column channel k has an associated parallel rinse channel k^* (**Figs. 33** and **34**). In other embodiments, there is only one rinse channel to provide rinsing for the entire set of row channels $\{j\}$, or there is any combination of rinse channels $\{k^*\}$ interspersed within the array

of column channels *{k}*. When there is only one rinse channel, it may be preferred to have it located adjacent to the last column channel of the array, where $k = n$.

Figure 35 shows, in a case of fluid flow through well *(j,k)*, an example of a method to rinse a segment of liquid or vapor from row channel j and into rinse channel k^*, where k^* is immediately adjacent to column channel k. This procedure and architecture allows this fluid segment to be directed to the waste reservoir without passing over wells *{(j, k+1), (j, k+2), etc.}* that are downstream along row channel j. This is desirable to limit the diffusion of a given compound from one well *(j,k)* to other wells downstream *{(j, k+1), (j, k+2), etc.}* where the compound may not be required. Additionally, as described below, diffusion may be controlled by introducing vapor segments into the microchannel microwell array.

Figure 32 shows how the pumping and valving scheme of Fig. 29, employing positive pressure source reservoirs, can be extended to work with a crossed array of n row channels and $2n$ column channels (n ordinary column channels plus n rinse column channels). This requires a $2n$ x 1 output valve manifold (420). In general, for every additional rinse channel k^* that is added, one additional valve is added to the valve manifold connected to the column channels. Similarly, the scheme of Fig. 30, employing a negative pressure waste reservoir, can be extended to work with an array incorporating rinse channels (130).

Dual-pumped designs

Flow through a fluid pathway {(j,k), (j,n), (m,k), (m,n) ... etc.} or more generally through any length of tubing is caused by a continuous pressure gradient that exists along the length of the pathway or tubing. Control of this pressure gradient by means of pressures applied at the inputs and outputs of the fluid pathways requires that there not be leakage of flow across the walls of the pathway. Leakage also compromises the proper dosing of compounds to the wells for drug screening. The sealing of the walls of the pathways must be adequate for the gauge pressures that are likely to be applied. For fluid flow rates of roughly a few millimeters per minute, path lengths not greater than a few centimeters, and channel cross sections on the order of 100 mm x 100 mm, the Hagen-Poiseuille law predicts that the required end-to-end pressure differential ΔP is less than 1 atmosphere (1 atm. $\approx$ 14 pounds per square inch). Nevertheless, considering that various materials and structures known in the art can be implemented for the device of the invention, the possibility of leakage between

neighboring wells is minimized by reducing the gauge pressure exerted across the walls of the system. Towards this end, the present device can use positive pressure source reservoirs with a negative pressure waste reservoir (i.e., a "double-pumped" system) to essentially cut in half the maximum gauge pressures which must be applied to achieve a given total end-to-end pressure differential.

For a "single-pumped" system, i.e., a system that is open to atmospheric pressure at one end and that is pumped at the other end (either to a positive or to a negative gauge pressure, $\pm\Delta P$), the pressure difference exerted across the walls of the system is a maximum at the location of the pumped reservoir, i.e., (200) of **Figs. 27** and **29;** or (320, 340) of **Figs. 28 and 30.** Leakage is most likely to occur at these points of maximum gauge pressure, with positive pressure tending to expel fluid out of the pathway and negative pressure tending to draw fluid into the pathway from a neighboring well or channel. (This assumes that the neighboring channels and wells are held at zero gauge pressure when not subjected to flow. Another method to control the pressure differentials across the system walls is to apply matching pressures to the neighboring pathways while they are not under flow.) At intermediate points along the fluid pathway, the pressure differential across the system walls has values between $\pm\Delta P$ and zero for positive and negatively pumped systems, respectively.

The same end-to-end pressure differential ΔP is exerted across the pathway by establishing pressures $\pm\Delta P/2$ at the input and output reservoirs, respectively. In this case, the maximum gauge pressures exerted across the walls of the system are $\pm\Delta P/2$ and occur at the corresponding endpoints of the system. Moreover, the applied gauge pressure within the device is close to zero if the wells are roughly at the midpoint between the input and output reservoirs along the length of the pathway. At the endpoints of the pathway, the maximum pressure differences $\pm\Delta P/2$ are more readily held without leakage within plastic tubing or silica capillaries. Detection of leakage is facilitated because it is likely to occur only at the system endpoints.

In one embodiment, the two syringes of a dual pumping system are the same diameter and volume, and because they are linked to the same actuator in a push-pull configuration, each syringe is moved the same linear distance, and identical volumes are exchanged for each increment of movement. As any particular source compound is pushed into the array, an equal volume of waste fluid is pulled out. Thus, the array of m source syringes also contains an array of m negative pressure waste reservoirs. This array of negative pressure waste

reservoirs is multiplexed to the column channels by a *1 x m* valve-less manifold, just as the array of source reservoirs is multiplexed to the row columns.

In another embodiment, the source and waste syringes are of different diameter, and the syringes must be controlled by two separate drivers that drive each syringe at a different linear rate, but yield the same volume changes in each syringe. For example, a smaller diameter syringe could be used for a compound, while a larger diameter syringe could be used as the waste receptacle. If the diameter ratio is a factor of 2, as the driver of the small syringe moves one linear unit to expel fluid, the driver of the large syringe moves only 1/4 linear unit to withdraw fluid, but the volumes exchanged are identical. **Figure 31** shows one particular case of this embodiment of a "dual-pumped" system, in which there is only one waste syringe pump that must be actuated in coordinated fashion (e.g., by means of computer control) with the actuation of any particular source syringe pump. Alternatively, the negative pressure pump may be a capillary action pump, also shown in **Fig. 31**. For the capillary action pump, the "actuation" of the pump is achieved by means of valves.

In summary, the dual-pumped system has the following advantages:

1) reduction in the maximum applied gauge pressure,
2) the applied gauge pressure is small near the midpoint of the pathway, and
3) leakage is readily detected and most likely to occur at the system endpoints.

Higher-level multiplexing

The use of multi-port flow manifolds between the pumped reservoirs and the crossed channel array allows *m* different pumps (200) or reservoirs (220) in these various embodiments, with *m* different liquid or vapor mixtures to selectively and sequentially produce flow in each well *(j,k)* of the *n x n* well array **(002)**. More generally, a set of wells defining a single fluid pathway {(j,k), (j,n), (m,k), (m,n) ... etc.} can be defined by this system. Flow of only one liquid or vapor mixture can occur through only one fluid pathway at a time. Higher level multiplexing, allowing simultaneous flow of different liquid or vapor mixtures through different pathways, is possible with a more complex system of flow manifolds. Such a system is easily constructed as a generalization of the present design.

Control of diffusion

The principle of this embodiment is that the valve element (520) and the valve seat (540) are shaped to allow flow in one direction and to suppress diffusion when flow is stopped.

Furthermore, the restoring forces on all valves are applied "passively" and constantly. No detailed control system for the check valves is needed, because the control of fluidic pressure gradients is the means by which individual valves are opened.

The use of crossed microchannels with a large number of open pathways between, for example, the **2n** microchannels and the $\mathbf{n}^2$ wells, may in some embodiments of the invention be augmented by additional methods to control the diffusion of compounds between wells and microchannels. Unlike prior art systems, the microfluidic system of the present invention does not need a detailed control system within the device for control of well-to-well diffusion. Two exemplary means of controlling inter-well diffusion are disclosed that do not require the active control of on-board valves. Both of these methods block inter-well diffusion by means of fluid flow control from valves, pumps, and manifolds that are external to the array of wells, and thus conform to the overall design principles of the invention: the optimal imaging of sub-arrays of wells in a class of fluidic architectures that requires fewer channels and permits smaller inter-well spacings.

The first method uses the insertion of segments of air between different fluid segments. The positive and/or negative pressure methods given in the above embodiments are employed to insert these air segments. In general, the application of differential pressure in appropriate sequences across a particular rows {j} and columns {k} or {k*} can place a number of separate air segments in the input channels {j} between a number of different fluids. In particular, appropriate sequencing can direct one fluidic segment to one well **(j,k)** and direct another fluidic segment to another well **(j, k-1)**, while also inserting and maintaining an air segment in row **j** between these two wells. Subsequently, the air segment can be passed out of the system through an unused well or via a rinse channel **k*** (130)(**Fig. 35**).

The second method of diffusion control uses "normally-closed" check valves (500) between every pair of adjacent vias (via **k** and via **k** + 1) (180) that connect the channels **j** to the wells **(j, k)** and **(j, k + 1)** (**Fig. 36**). Similarly, check valves may be used in architectures incorporating rinse column channels *{k*}*. In other embodiments, check valves may be

placed within the vias (160, 180) that connect wells to microchannels or row channels to rinse column channels. These valves are closed unless a pressure gradient is applied across a fluid pathway of the array. Then only those valves distributed along the selected fluid pathway are pushed open. When flow is stopped and the valves return to the closed position, the valves suppress diffusion of compounds between wells and microchannels, or row channels and rinse channels. The restoring force that closes each valve element (520) in its corresponding valve seat (540) may be provided by any number of methods known in the art. The strength of the restoring force must allow the valves to open upon the application of pressure gradients that yield appropriate flow rates (typically less than 1 cm/min.). Methods for application of restoring forces include mechanical force by the deformation of metallic, semiconductor, or organic materials; pneumatic pressure; electrostatic force, magnetic force, and the force of gravity.

In another embodiment, an externally-applied magnetic field gradient (620) induces a restoring force on valve elements (520) that incorporate a magnetic material. This includes but is not limited to the use of paramagnetic beads (600) (e.g., supplied by Dynal Corp.) as shown in **Fig. 37**. These paramagnetic beads (600) serve as balls in micro ball valves that are fabricated within and along each row and column channel. U.S. Patent Nos. 5,643,738 and 5,681,484 describe a magnetically-actuated microball check valve. However, these patents particularly describe an actively-controlled valve that would permit closure of the ball valve "on demand," and has as its purpose to stop flow that is imposed by some external pressure. In the instant case, the particular advantage to the microfluidic architectural system is that a mild restoring force is simultaneously applied to a large ensemble of ball valves throughout the array, and no active control is required. The movement of selected balls (whenever it is desired to open the valves) along a fluid pathway is achieved by means of causing the fluid itself to flow. The ball valve is controlled by the flow of fluid, not the flow of fluid is controlled by the ball valve. Moreover, in this embodiment, the valve seat is particularly designed so that a seal is not obtained in one direction, as opposed to the other direction, where a mild seal sufficient to stop diffusion is obtained.

In one embodiment, one micro ball valve is located between every pair of adjacent vias (via k and via k + 1) that connect the channels **j** to the wells **(j, k)** and **(j, k + 1)**. Each micro ball valve opens only when a pressure gradient is applied across a pathway that includes that valve. The design of the valve seat (540) on one side is shaped (e.g., with

grooves) so that flow is not stopped when fluidic pressure moves the ball in the direction of flow. When no pressure gradient is applied (and no fluid is flowing) at the site of a particular ball valve, a magnetic restoring force presses that paramagnetic ball back against the other side of the valve seat (540)(where the seat is shaped to fit the ball) to suppress diffusion of compounds across that portion of the microchannel. This restoring force is induced by a magnetic field gradient applied to the entire array, so that all valves are normally closed.

In another embodiment, an externally-applied magnetic field gradient (620) induces a restoring force on valve elements (520) that incorporate a ferromagnetic material. For example precision chrome steel balls are available from Glenn Mills Inc., Clifton, NJ in a range of sub-mm to few mm diameters.

Fig. 38 shows an embodiment of a shaped valve seat that exemplifies this design principle. Specifically, diffusion past the ball is stopped when the externally-applied restoring force presses the ball to the left into a round opening in the valve seat. When flow is actuated by externally-applied pressures, the ball is forced to the right, but the ball does not stop the flow because the right side of the valve seat is oval shaped and includes a groove-like deformation at along one side, forming path for fluid to bypass the ball in this open position of the valve.

Fig. 39 shows an embodiment that allows a single magnetic field gradient applied to the whole cassette of the invention to induce force on both a set of ball valves of the input channels and a set of ball valves of the output channels

In one embodiment the direction of the magnetic field gradient is parallel to the plane of the array, but oriented at 45 degrees to both the row and column channels. Thus each ball experiences a component of force in the direction of the "closed" side of its corresponding valve seat (540). In other embodiments, the magnetic field gradient is applied perpendicular to the plane of the array, and the ball valves operate in the vertical direction within the horizontal microchannels or within the vertical vias. In the latter case (vertically-actuated ball valves within vertical vias) the via architecture must again allow each ball to be subjected to a component of magnetic force in the direction of the "closed" side of its corresponding valve seat (540).

An example has been given of a ferromagnetic or paramagnetic valve element (520) of spherical shape, but other shapes and other restoring forces may generally be used that function in the same manner.

Well dimensions and characteristics

In another aspect, the present invention provides a cassette for cell screening, comprising a substrate having a surface, wherein the surface contains a plurality of cell binding locations; a fluid delivery system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the substrate, wherein the multi-level chamber comprises a crossed array of microfluidic input channels and output channels and a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels; and a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location, and wherein the wells are present at a density of at least about 20 wells per square centimeter. In a preferred embodiment, the well density is between about 20 wells per square centimeter and about 6400 wells per square centimeter.

In a preferred embodiment, the cassette of this aspect of the invention further comprises one or more input manifolds in fluid connection with the microfluidic input channels one or more output manifolds in fluid connection with the microfluidic output channels. In another preferred embodiment, the cassette of this aspect of the invention further comprises at least one source receptacle in fluid connection with the one or more input manifolds and at least one waste receptacle in fluid connection with one or more output manifolds. The various control devices in these embodiments are as described above.

No limitation is placed on the well shape geometry in the instant invention, as the well shape may differ in different embodiments of the invention. While a rectangular well shape allows for the minimum distance and area between wells in the array, this shape has the disadvantage of potential differences in cell culture or fluidic properties in the corner regions of the wells. Therefore, a well shape that is circular or that is rectangular with rounded corners is used in a preferred embodiment of the invention.

The array system of the present invention is designed for the imaging of individual cells within a field of cells in a drug screening assay system. Screening with imaging assays that involve the measurement of each individual cell within a field of cells, in parallel, is referred to as High Content Screening (HCS), because detailed information about intracellular processes is contained within the image of a single cell. Screening with lower resolution imaging or with detectors that integrate a signal over a population of cells is

referred to as High Throughput Screening (HTS), because it is faster. In HCS, the cells are typically identified by imaging the nuclei of the cells, which are spherical or ellipsoidal in shape with a long-axis length that ranges typically from 5 μm to 15 μm. The cell array used in the present invention optimizes the rate of HCS by using a higher-density multi-well format compared to conventional 96-well plates.

For HCS, the number of cells that provide a statistically-relevant sample as well as the number of cells that are required per unit surface area varies depending on the type of assay and the type of cell being cultured. Thus, the minimum size of the well required by statistical criteria for each assay is different. Nevertheless, no matter what that minimum or optimum well size is, the rate at which wells can be read with adequate image resolution to resolve individual cell nuclei is optimized by the present device, which allows several wells to be imaged at one time in parallel. This is done most efficiently if there is minimal wasted space between wells, and the cell-plated area within the entire image is therefore maximized.

Moreover, the present array system optimizes the flexible implementation of both HTS and HCS on the same optical imaging cell-based screening system. The required pixel density (pixels per micron) is lower for HTS (for example, 0.001 to 0.1 pixels per micron) and higher for HCS (for example, 0.1 to 4.0 or more pixels per micron). The present system provides seamless, combined use of HTS and HCS, where a "hit" identified in HTS is also read in HCS mode for more detailed analysis. In fact, the availability of HCS on the same platform and with the same sample, greatly increases the information associated with "hits" identified in the HTS mode. Nevertheless, the fastest possible rate of HCS is advantageous to allow maximum overall screening throughput whenever HCS is used. The minimum pixel density required for HCS in combination with the maximum well density of the array are the two critical parameters that define the maximum rate at which wells can be read in the HCS mode. This invention describes how the design of the present array system can maximize this HCS rate, as well as the HTS rate.

In use with 96-well microplates, a conventional microscope imaging system can image square fields that are 0.1 mm to 10 mm wide centered on each 7-mm diameter well. The wells are imaged or "read" in <u>series</u>. At each position of the stage, typically, only one area of a well is read. To read the entire array (either in HTS or HCS modes) the sample stage must move the microplateat least 96 times.

In this invention, we define a novel cell screening method in which many wells are read at one time. Because the present array has well widths and inter-well distances that are roughly 10 times smaller than in the 96-well microplate (wells that are sub-millimeter in width and overall plate dimensions of several millimeters rather than several centimeters wide), a cell screening system, such as that disclosed in the present invention, can image many wells simultaneously. Thus, the sample stage is moved fewer times per plate and several wells are read in parallel (Fig. 39).

This essentially involves the serial imaging of "sub-arrays" (020) of the entire microwell array (040), while acquiring data from all wells of each sub-array in parallel. For example, the imaging of 3 x 3 well sub-arrays requires 9 times fewer movements of the sample stage, and allows a roughly 9-times faster rate of data acquisition.

For a given CCD detector array (080), the magnification of the optical system (060) determines the area of the sub-array (020) that is projected onto the detector. For example, an image pixel density of 1 pixel per micron is achieved for a 1000 μm x 1000 μm sub-array of wells by using 10x optical magnification and a 1000 x 1000 pixel CCD array of 10 μm x 10 μm elements. In a second example, a 2000 μm x 2000 μm sub-array of wells can be imaged onto a 2000 x 2000 pixel CCD array at the same magnification to yield the same image pixel density. In a third example, an image pixel density of 0.1 pixel per micron is achieved for a 10mm x 10mm sub-array of wells by using 1.0x optical magnification and a 1000 x 1000 pixel CCD array of 10 μm x 10 μm elements. In a fourth example, a 10mm x 10mm sub-array of wells can be imaged onto a 2000 x 2000 pixel CCD array at 2.0x magnification to yield the same image pixel density.

Below we give embodiments of particular embodiments of well sizes and spacings according to the present invention that optimize the imaging of sub-arrays. These well sizes must be large enough to contain a desired number of cells bound to a desired number of cell binding sites within each location of the array. Due to the considerable range of desired cell densities to be cultured on the cell binding sites (i.e.: cultures of very widely spaced cells, or of confluent monolayers may be desired), the desired well size may range from that which contains only one cell on a very small cell binding site of about 10 μm in diameter (i.e.: a well size of 20 to 50 microns), to that which contains either one large cell binding site or an array of many cell binding sites (i.e.: a well size of 1 to 2 mm) comprising a single location of

the array. The former case leads to higher well densities, and the latter to lower well densities.

For both high and low well densities, and for wells that contain one or many cell binding sites, the class of microfluidic architectures according to the present invention permit the optimal spacing of wells, the minimal wasted space between the wells, and the maximum number of wells that can be simultaneously imaged in a sub-array and still obtain adequate pixel resolution in the image.

We now use the four examples of optical resolution given above to illustrate how for a preferred range of sub-array sizes , optical magnifications, and pixel resolutions, and based on the advantages of the microfluidic architecture described herein, the present device supports well sizes and spacings that enable great increases in the speed at which the wells can be imaged or read. Additional sub-array sizes, optical magnifications, and pixel resolutions are supported by the methods and devices of the invention. The scope of the invention is not limited by the current state of the art in the number of available pixels, nor in the pixel sizes in electronic imaging systems.

1. For the first example above, a 1000 μm x 1000 μm sub-array of wells is imaged onto a 1000 x 1000 pixel CCD camera (with image resolution of 1 pixel per micron). We now give four examples of well sizes and densities that are supported by this invention. (a) First, a 2 x 2 sub-array of 300 μm x 300 μm wells with 200 μm-thick walls and a well density of 400 wells per cm^2. Imaging these wells in groups of 4 yields a 4-fold speed increase compared to reading the plate one well at a time. (b) Second, a 3 x 3 subarray of 200 μm x 200 μm wells with 100 μm walls yields a 9-fold increase in speed and a well density of 1111 wells per cm^2. The number of wells per unit area is a factor of 80 greater than that provided by the current highest density commercial microplate (the 1536 well plate). (c) Third, a 5 x 5 sub-array of 100 μm x 100 μm wells and 100 μm walls yields a 25-fold speed increase and a density of 2500 wells per cm^2. (d) Fourth, for an even higher density of wells and a greater speed advantage, the well can be 25 μm x 25 μm with 100 μm walls, yielding an 8 x 8 sub-array, a 64-fold speed increase, and a well density of 6400 cells per cm^2.
2. For a 2000 μm x 2000 μm sub-array of wells imaged onto a 2000 x 2000 pixel CCD camera (with image resolution of 1 pixel per micron), one additional example of well

density is considered in addition to the four examples considered above. (a) First, a 2 x 2 sub-array of 800 μm x 800 μm wells with 200 μm-thick walls yields a 4-fold speed increase and a well density of 100 wells per cm^2. The number of wells per unit area is a factor of 5 greater than that provided by the current highest density commercial microplate (the 1536 well plate). (b) Second, a 4 x 4 sub-array of 300 μm x 300 μm wells with 200 μm-thick walls yields a 16-fold speed increase and a well density of 400 wells per cm^2. (c) Third, a 6 x 6 sub-array of 200 μm x 200 μm wells with 100 μm walls yields a 36-fold speed increase and a well density of 900 wells per cm^2. (d) Fourth, a 10 x 10 sub-array of 100 μm x 100 μm wells with 100 μm walls yields a 100-fold speed increase and a well density of 2500 wells per cm^2. (e) Fifth, a 16 x 16 sub-array of 25 μM x 25 μM wells with 100 μm walls yields a 256-fold speed increase and a well density of 6400 wells per cm^2.

3. For a 10 mm x 10 mm sub-array of wells imaged onto a 1000 x 1000 pixel CCD camera (with image resolution of 0.1 pixel per micron), we again describe the four cases of well sizes and densities described in example 1 above. (a) First, a 20 x 20 sub-array of 300 μm x 300 μm wells with 200 μm-thick walls yields a 400-fold speed increase and a density of 400 wells per cm^2. (b) Second, a 30 x 30 sub-array of 200 μm x 200 μm wells with 100 μm walls yields a 900-fold speed increase and a well density of 1111 wells per cm^2. As before, this case supports a well density 80 times greater than that provided by the current highest density commercial microplate. (c) Third, a 50 x 50 sub-array of 100 μm x 100 μm wells with 100 μm walls yields a 2500-fold speed increase and a well density of 2500 wells per cm^2. (d) Fourth, an 80 x 80 sub-array of 25 μm x 25 μm wells with 100 μm walls yields a 6400-fold speed increase and a density of 6400 wells per cm^2 .For an even higher density of wells and a greater (2500 fold) speed advantage, the wells can be 100 μm x 100 μm with walls separating the wells that are 100 μm wide, yielding 2500 wells per sub-array, and 2500 wells per cm^2.

4. For a 10 mm x 10 mm sub-array of wells imaged onto a 2000 x 2000 pixel CCD camera (with image resolution of 0.1 pixel per micron), the cases from example 2 above of 100, 400, 1111, 2500, and 6400 wells cm^2 again apply, but yield 100 times greater speed increases because the subarrays are 10 times wider on each side. Thus, the speed

increases for well densities of 100, 400, 1111, 2500, and 6400 wells/cm2 are 400X, 1600X, 3600X, 10,000X, and 25,600X, respectively, in the 10 mm x 10 mm array.

The following table compares well pitches for various other devices:

Type of plate	Reference	Well to well distance (mm)	Wells/cm^2	Estimated Speed increase*
Standard 1536 well plate		2.25	20	
12 x 12 array in 1.5" x 1.5" area	Gen. Engr. News **18:12** (1998)	3	11	
864 well plate: 24 x 26 array in std. microplate footprint of 108 cm^2	US 5,910,287	3	11	
9600 well plate: 80 x 120 array in a std. microplate footprint of 108 cm^2	US 5,910,287	0.9	123	
	Present invention			
1 mm x 1mm subarray: 1000 x 1000 pixel CCD	Example 1(a)	0.5	400	4X
	Example 1(b)	0.3	1111	9X
	Example 1(c)	0.2	2500	25X
	Example 1(d)	0.125	6400	64X
2 mm x 2 mm subarray: 2000 x 2000 pixel CCD	Example 2(a)	1	100	4X
	Example 2(b)	0.5	400	16X
	Example 2(c)	0.3	1111	36X
	Example 2(d)	0.2	2500	100X
	Example 2(e)	0.125	6400	256X
10 mm x 10 mm subarray: 1000 x 1000 pixel CCD	Example 3(a)	0.5	400	400X
	Example 3(b)	0.3	1111	900X
	Example 3(c)	0.2	2500	2500X

	Example 3(d)	0.125	6400	6400X
10 mm x 10 mm subarray: 2000 x 2000 pixel CCD	Example 4(a)	1	100	400X
	Example 4(b)	0.5	400	1600X
	Example 4(c)	0.3	1111	3600X
	Example 4(d)	0.2	2500	10,000X
	Example 4(e)	0.125	6400	25,600X

*Estimated speed increase is a comparison of the speed at which the entire array could be imaged by sub-arrays compared to imaging by separately imaging each well

All of these particular aspects of the instant microfluidic device – the crossed-channel, multilevel architectures, the high well density, the fluid flow control into these architectures, the arrangement of wells into spatially-optimized sub-arrays of wells, and the optional means of control of inter-well diffusion of compounds – are specifically selected and designed to form an integrated system that is compatible with the use of cell culture medium (a polar, aqueous solution containing biological macromolecules and salts), with the maintenance of desired, physiological levels of dissolved oxygen and carbon dioxide gases in the medium, and with the maintenance of desired, physiological temperature (typically 37° C, but other temperatures in the approximate range of 15° C to 40° C may be desired for particular cell types).

Particularly, the cell culture medium may be equilibrated in off-board vessels to the desired levels of temperature and dissolved carbon dioxide and oxygen. Then, using off-board vessels and valves, the medium is moved through the microchannels and to the wells. The sealing of these microchannels and wells from the atmosphere enables the partial pressures of gasses to be controlled by means of the equilibration of the external vessels. In a preferred embodiment, the level of carbon dioxide in the media within the wells may be established and maintained by equilibrating the media prior to its flow into the device, and/or by allowing exchange of a mixture of carbon dioxide and air with the media as it sits within the well. A number of different environmentally controlled chambers for live cell culture exist. (U.S. Patent Nos. 5,552,321 and 4,974,952; Payne et al., J. Microscopy

147:329-335 (1987); Boltz et al., Cytometry 17:128-134 (1994); Moores et al., Proc. Natl. Acad. Sci. 93:443-446 (1996); Nature Biotech. 14:3621-362 (1996)).

In a further preferred embodiment, the temperature of the entire substrate is controlled by being in contact with, or composed integrally with, a heater and with a temperature sensor. An electronic temperature control system regulates the heater to attain a chosen temperature set point. Thus, the design of the microfluidic array system described in this invention is readily used in a way that supports live cell culture without an external incubator system. **Figure 12** shows how this invention provides for the automated reading of cassettes. The cassettes are kept under controlled environmental conditions within two storage compartments (**48** and **54**) before and after reading in the luminescence reader instrument **44**. While being read by the luminescence reader instrument, the system maintains the appropriate temperature and composition of dissolved gasses in the cell culture media within the wells of the cassette. Temperature control within the wells is provided by heating device(s) and temperature sensor(s) within the cassette of the luminescence reader instrument. Control of dissolved gas composition within the wells is provided by equilibration of the media with a source of pre-mixed air and carbon dioxide (available commercially) prior to the flow of media into the wells of the cassette. Any type of controller for regulating gas partial pressures may be used with the instant invention. In a preferred embodiment, the system comprises a gas controller comprising a pre-mixed gas source connected to a reservoir or reservoirs containing cell culture media and/or test compounds. A gas pressure regulator and flow control valve control the rate of flow of this gas mixture and its pressure in the connected fluid reservoir(s). This equilibration of the gas mixture with the fluids may be carried out in reservoirs either on-board or off-board the cassette, but in any case, outside the matrix of the wells.

The present invention fulfills the need in the art for devices and methods that decrease the amount of time necessary to conduct high throughput and/or high content cell-based screening. The devices of the invention are also ideally suited as a cell support system for a hand-held diagnostic device (i.e.: a miniaturized imaging cell-based assay system). The drug discovery industry already uses 96-well microplates and is in transition towards the use of 384-well plates. Plates with up to 1536 wells are envisioned. Thus, there is a great advantage in both throughput and economy from the use of even higher density plates, such as that of the present invention.

The sealed containment of the cells in the cell array of the instant invention will provide a rugged system that is portable and usable in any orientation. For military and civilian toxin testing, the devices of the instant invention will provide two primary advantages. First, toxin testing based on cellular function is advantageous compared to toxin testing based on molecular structure (e.g., mass spectrometry or optical spectroscopies), because it tests the ultimate effect of the compound on tissue rather than a related property of the compound whose link to toxicity may be unknown or dependent on other conditions. Second, an automated, miniaturized, sturdy, and portable format for cellular-function based sensor would be advantageous compared to the current state-of-the-art that involves large microplates filled by large robotic pipetting systems and read by large microscopic readers.

Additionally, other assay capabilities can be integrated into the device of the present invention that are not possible in prior art devices, such as simple plastic microplates. For example, a mass spectrometric or capillary electrophoretic analytical device, or systems for DNA and/or protein analysis, can be integrated in the instant device to measure chemical and structural parameters of test compounds. Such integration would provide information that is complementary to the cell-based, functional parameters measured by a HCS or HTS system. For conventional microplates, such additional assay functions are external to the plate, and therefore require extensive additional equipment for both the transfer of samples from the microplate and for the analytical equipment itself. In the miniaturized format with integrated fluidics of the present invention, the sample is integrally pumped to a microanalytical system on the chip or on an integrally-connected second chip. Such microanalytical chips will reduce the cost and dramatically increase the speed of cell-based analysis. In this context, the marriage of a microscale live-cell analytical system with a microscale chemical analytical system is expected to provide great improvements over existing methods and devices.

We claim:

1. A cassette for cell screening, comprising:

a. a substrate having a surface, wherein the surface contains a plurality of cell binding locations;

b. a fluid delivery system for delivering assay components to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the substrate, wherein the multi-level chamber comprises

i. a crossed array of microfluidic input channels and output channels;

ii. a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels; and

c. a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location, and wherein the wells are present at a density of at least about 20 wells per square centimeter.

2. The cassette of claim 1 wherein the wells are present at a density of between about 20 wells per square centimeter and about 6400 wells per square centimeter.

3. The cassette of claim 1 wherein the multi-level chamber further comprises:

(i) one or more input manifolds in fluid connection with the microfluidic input channels; and

(ii) one or more output manifolds in fluid connection with the microfluidic output channels.

4. The cassette of claim 3 further comprising:

(i) at least one source receptacle in fluid connection with the one or more input manifolds;

(ii) at least one waste receptacle in fluid connection with one or more output manifolds.

5. A cassette for cell screening comprising

a. a substrate having a surface, wherein the surface contains a plurality of cell binding locations;

b. a fluid delivery system for delivering reagents to the plurality of cell binding locations, wherein the fluid delivery system comprises a multi-level chamber that mates with the substrate, wherein the multi-level chamber comprises

i. a crossed array of microfluidic input channels and output channels, wherein each well is in fluid connection with one or more input channels and one or more output channels;

ii. a plurality of fluidic locations in fluid connection with the microfluidic input channels and output channels;

iii. one or more input manifolds in fluid connection with the microfluidic input channels;

iv. one or more output manifolds in fluid connection with the microfluidic output channels;

v. at least one source receptacle in fluid connection with the one or more input manifolds; and

vi. at least one waste receptacle in fluid connection with one or more output manifolds; and

c. a plurality of wells, wherein an individual well comprises the space defined by the mating of one cell binding location and one fluidic location.

6. The cassette of claim 1 or 5 further comprising a pump to control fluid flow within the microfluidic device, wherein the pump is in fluid connection with the one or more input manifolds and the one or more output manifolds.

7. The cassette of claim 1 or 5 further comprising a temperature controller.

8. The cassette of claim 1 or 5 further comprising a controller for regulating gas partial pressure.

9. The cassette of claim 1 or 5 wherein the crossed array of microfluidic input channels and output channels is non-intersecting.

10. The cassette of claim 1 or 5 further comprising rinse channels in fluid connection with the output channels and the waste reservoir.

11. The cassette of claim 1 or 5 further comprising valves between adjacent wells or within the rinse channels.

12. The cassette of claim 11 wherein the valves comprise a magnetic element.

13. The cassette of claim 1 or 5 further comprising an integrated mass spectrometric or capillary electrophoretic analytical device, or a system for protein and/or DNA analysis.

14. An improved method of cell screening, wherein the improvement comprises providing the cassette of claim 1 or 5 to supply fluid to an array of locations which contains cell binding sites.

15. An improved method for diffusion control in a cassette, wherein the improvement comprises constantly applying a passive restoring force to valves located within microfluidic channels of the cassette.

16. A method for cell screening, comprising

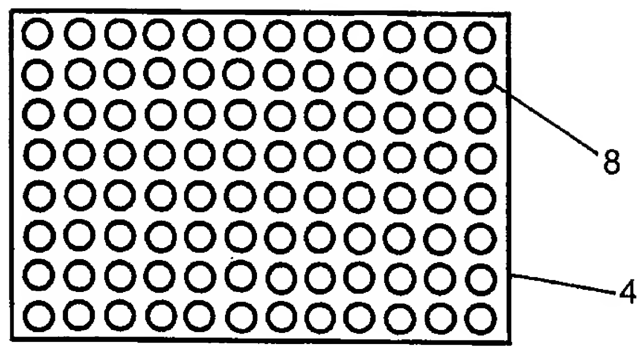
a) providing an array of locations which contain multiple cells;
b) providing an optical system to obtain images of the array of locations;
c) serially imaging sub-arrays of the array of locations; and
d) acquiring data from each of the sub-arrays in parallel.

17. A method for cell screening, comprising

a) providing the microfluidic device for cell screening of claim 1 or 5;
b) providing an optical system to obtain images of the array of locations;
c) serially imaging sub-arrays of the array of locations; and
d) acquiring data from each of the sub-arrays in parallel.

Figure 1

A



B

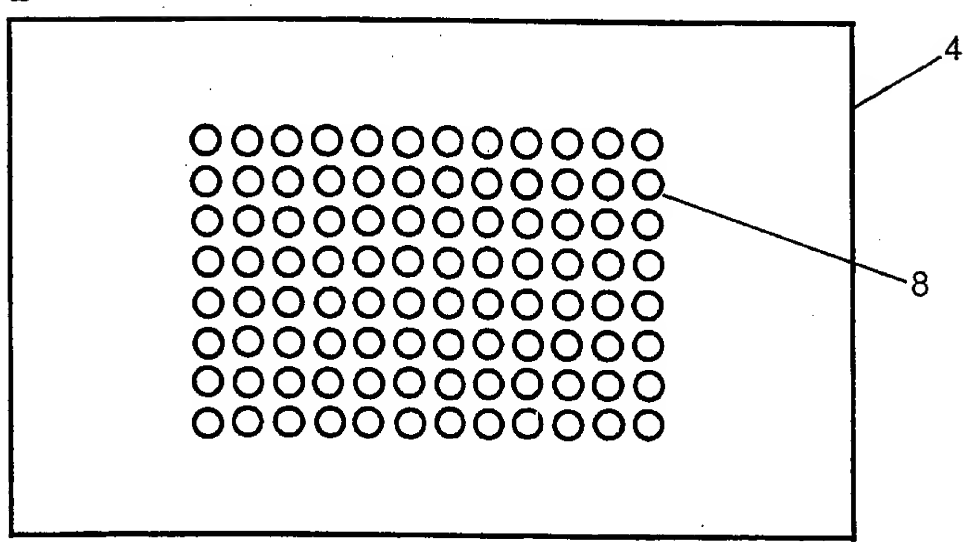


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Figure 2

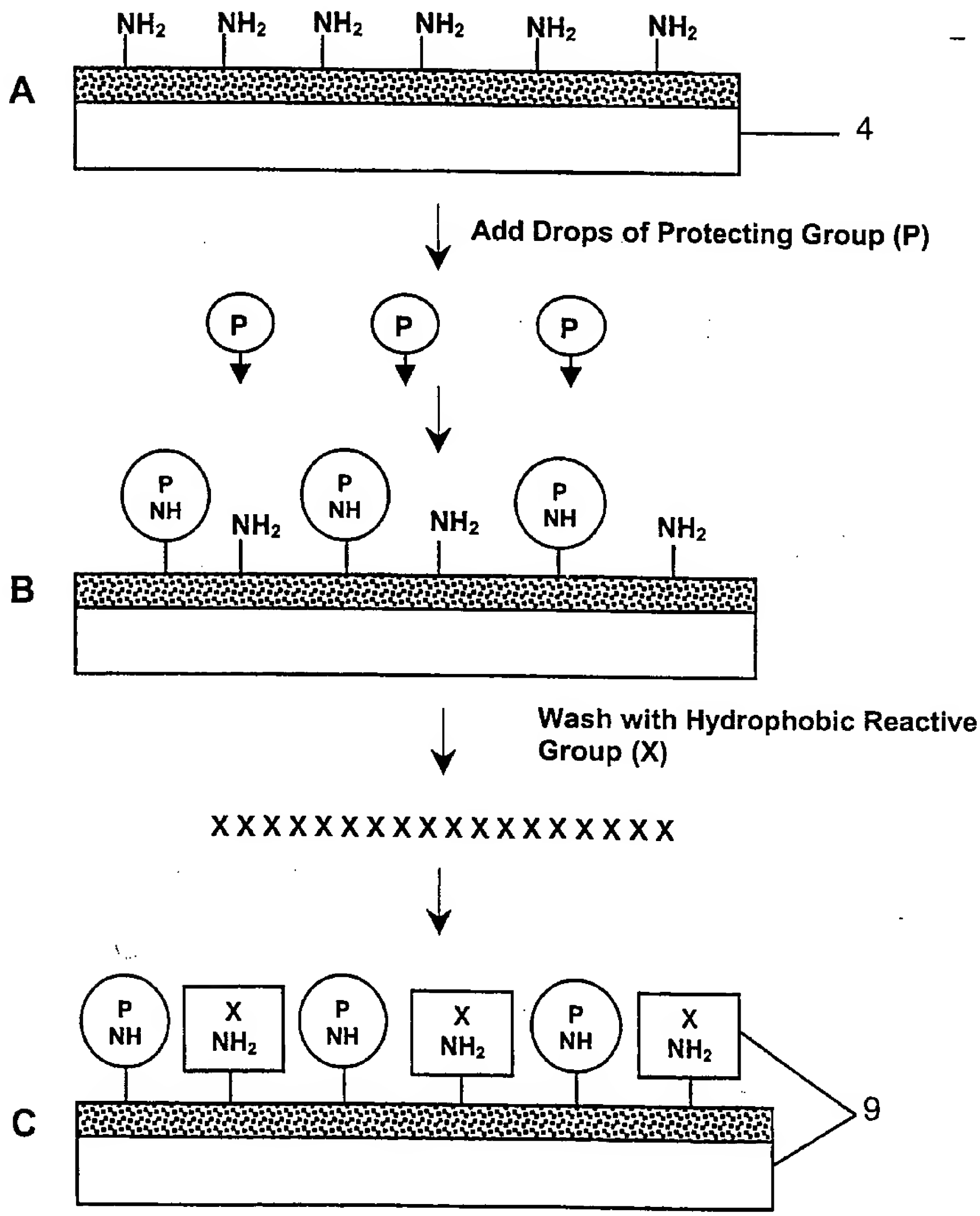

NH2
A
4
Add Drops of Protecting Group (P)
P
NH
B
Wash with Hydrophobic Reactive Group (X)
XXXXXXXXXXXXXXXXXXXX
X
C
9

Figure 3A

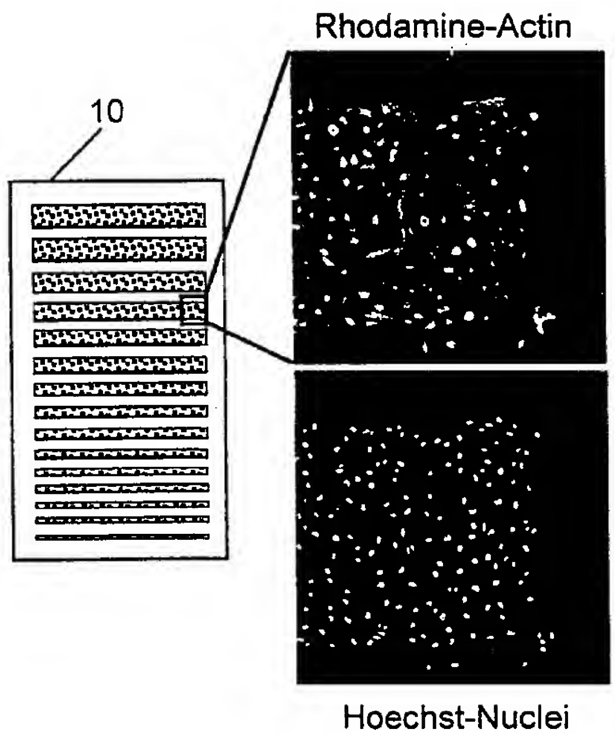
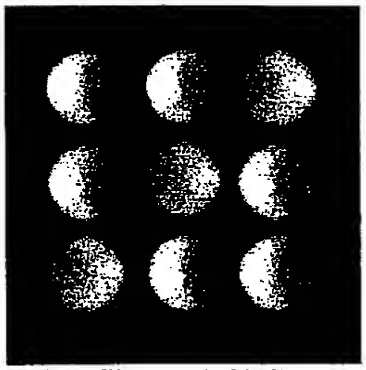
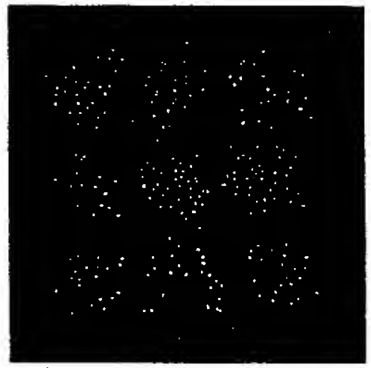


Figure 3B

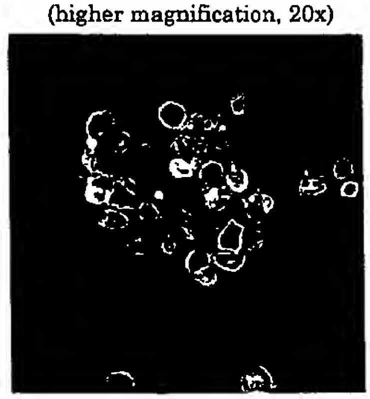


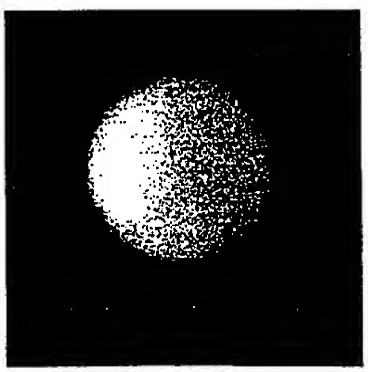
Sodium Fluorescein binding to Cell Chip to identify the location of 200 um spot patterns.



Fluorescent cell attachment of L929 cells to patterned spots (low magnification, 4x)

Fluorescent cell attachment of 3T3 cells to patterned spots. Nuclei (above), Actin in same cells (below) (higher magnification, 20x)





Sodium Fluorescein binding to Cell chip to identify the location of 400 um spots patterns.

Figure 4

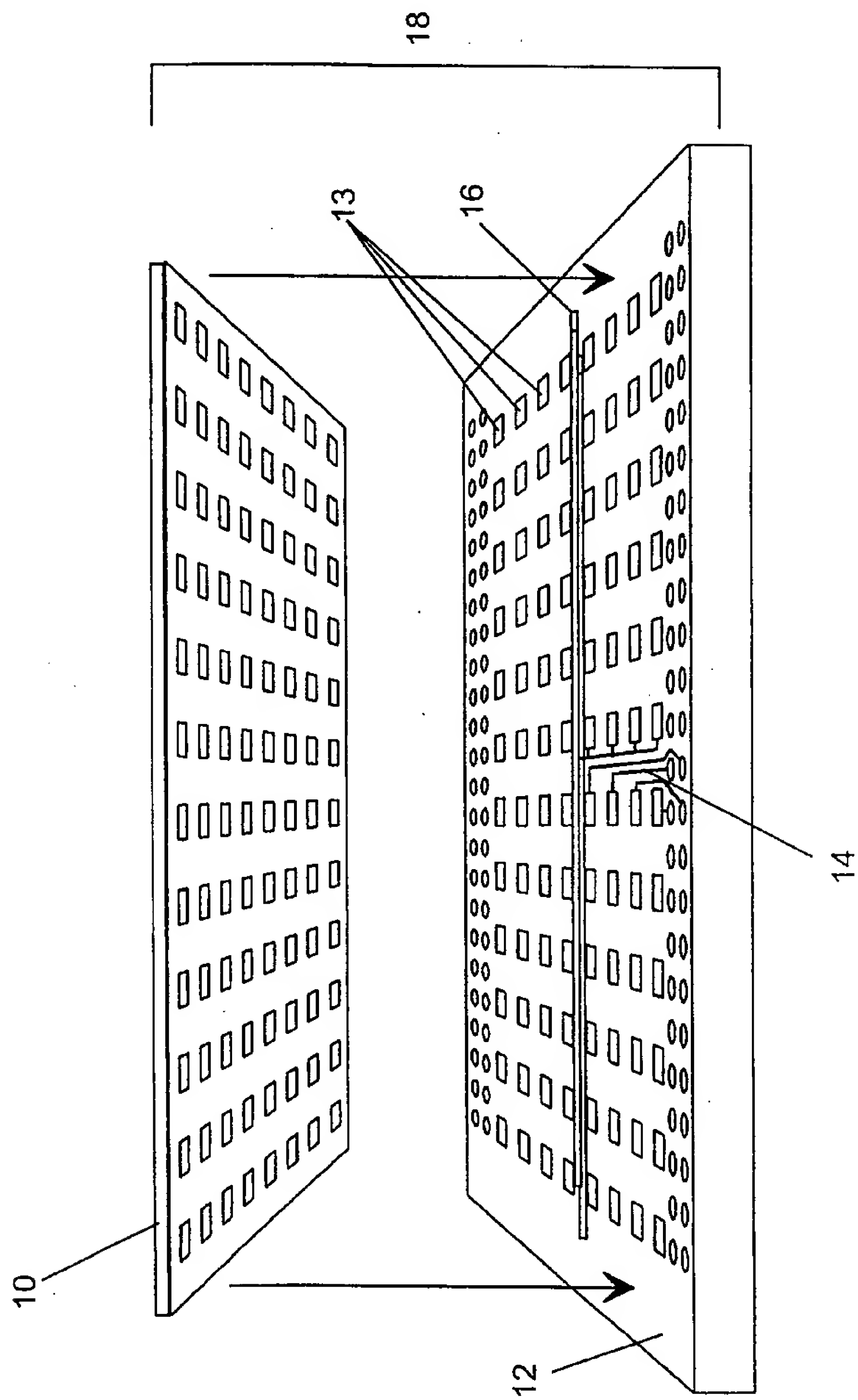


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Figure 5

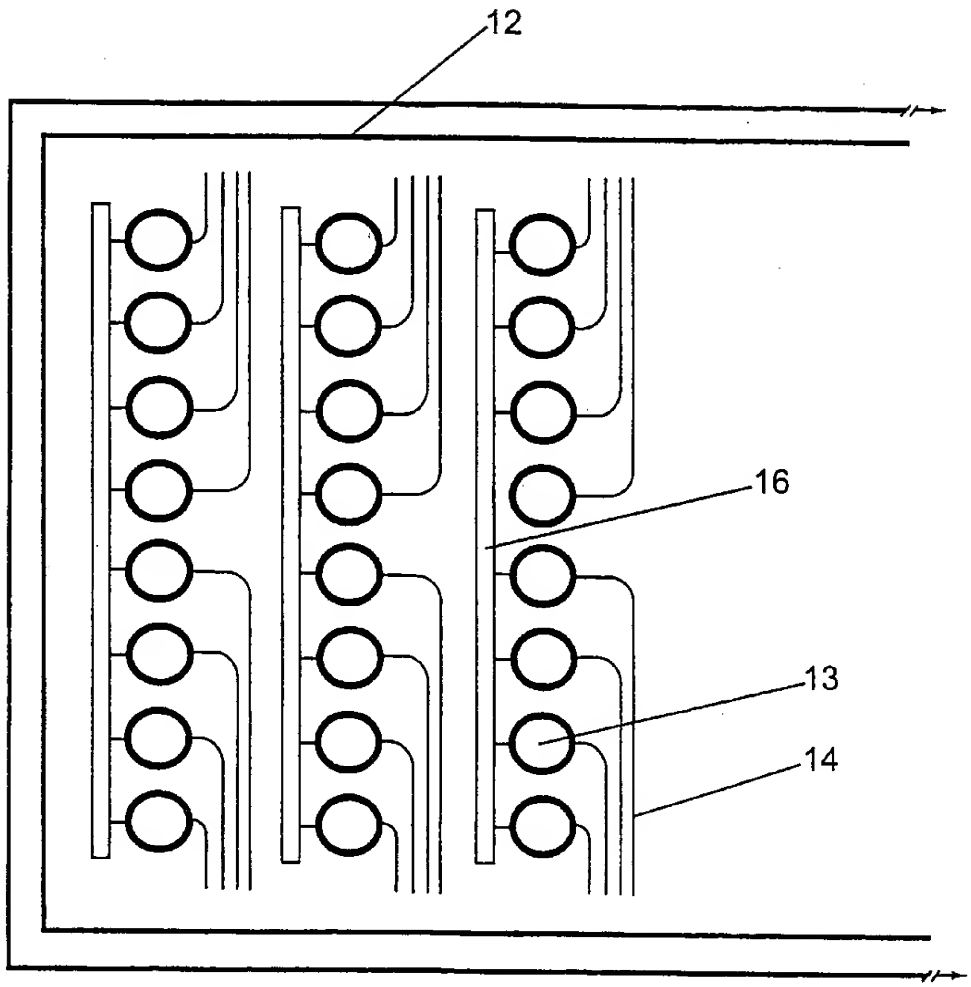

Figure 6

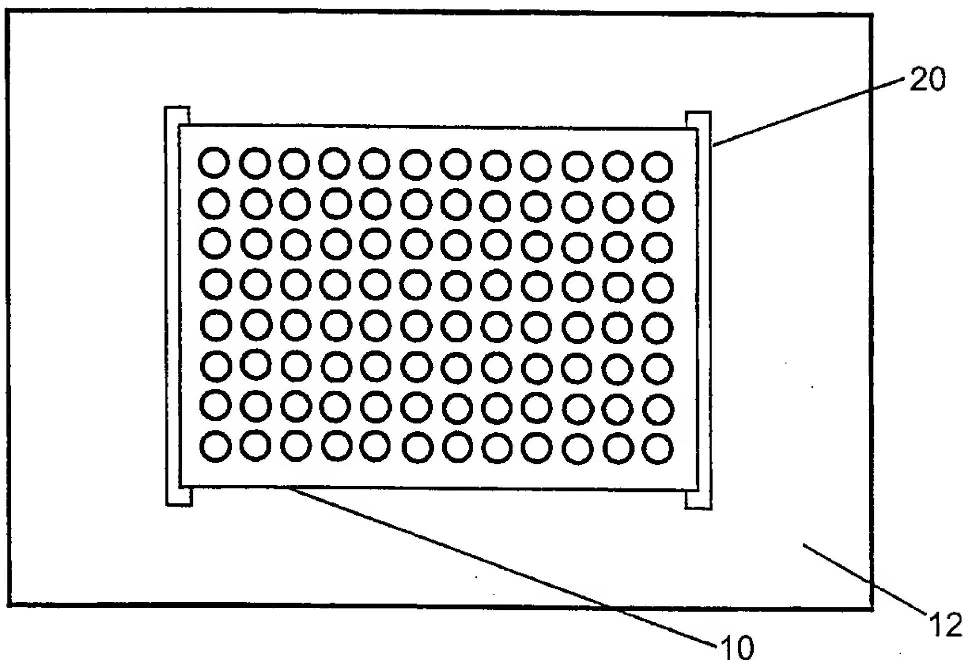

Figure 7

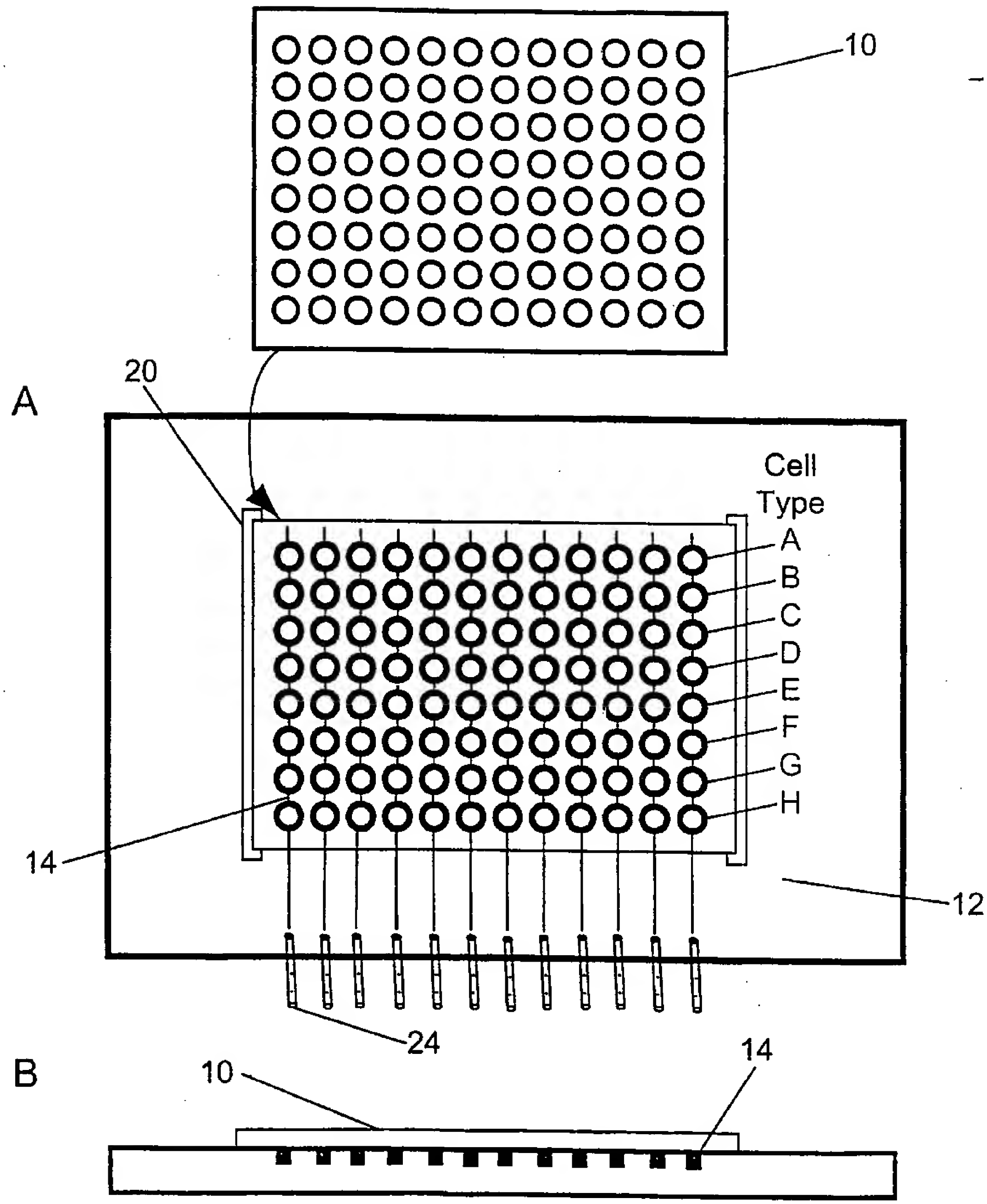

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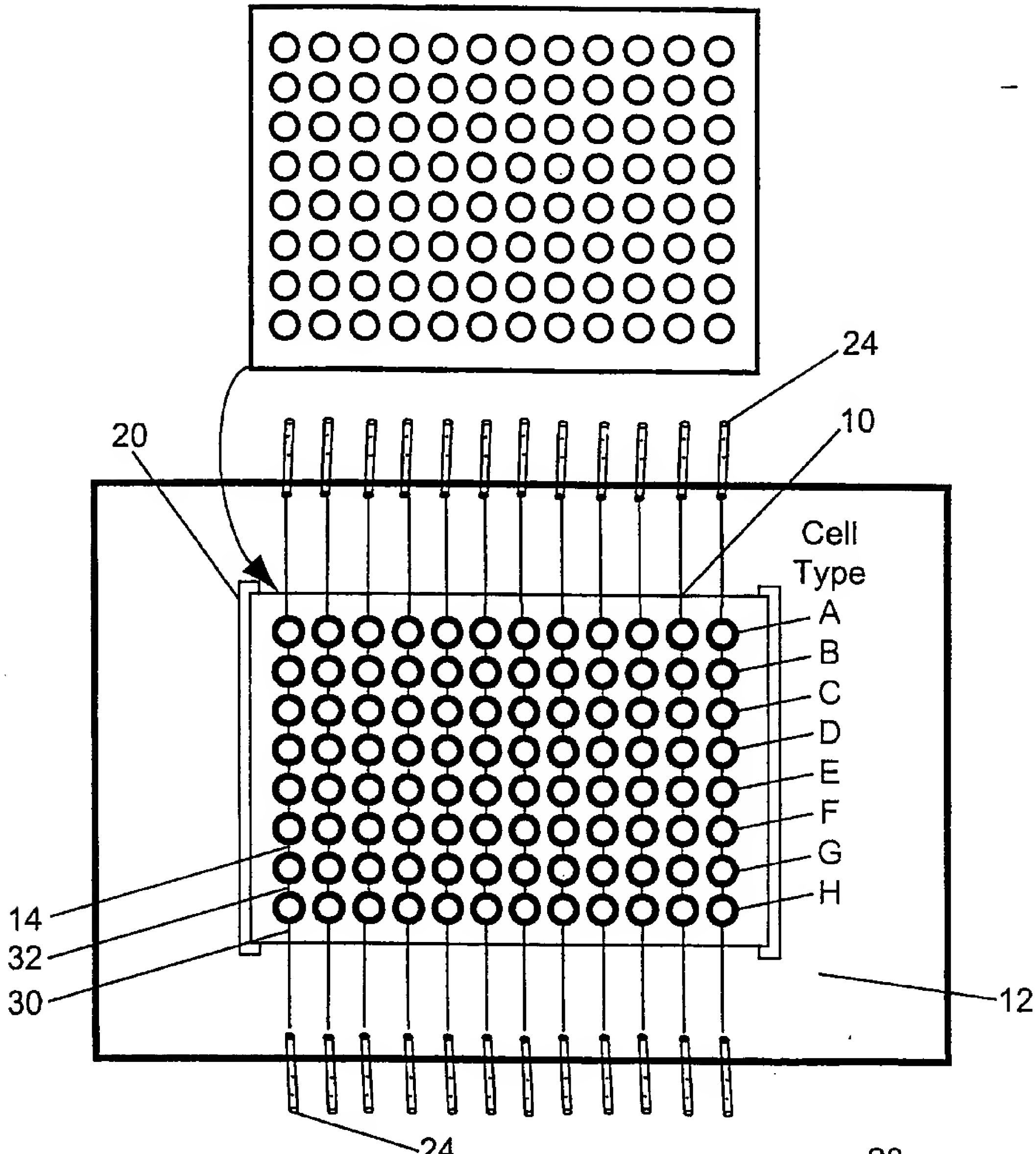
Figure 8

A



B

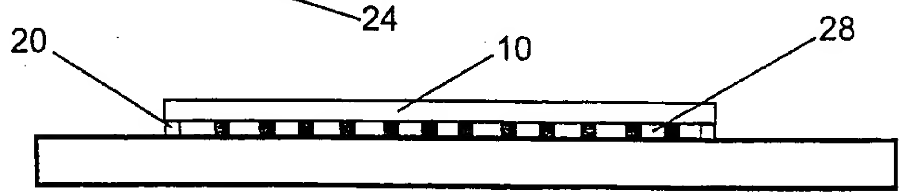


Figure 9

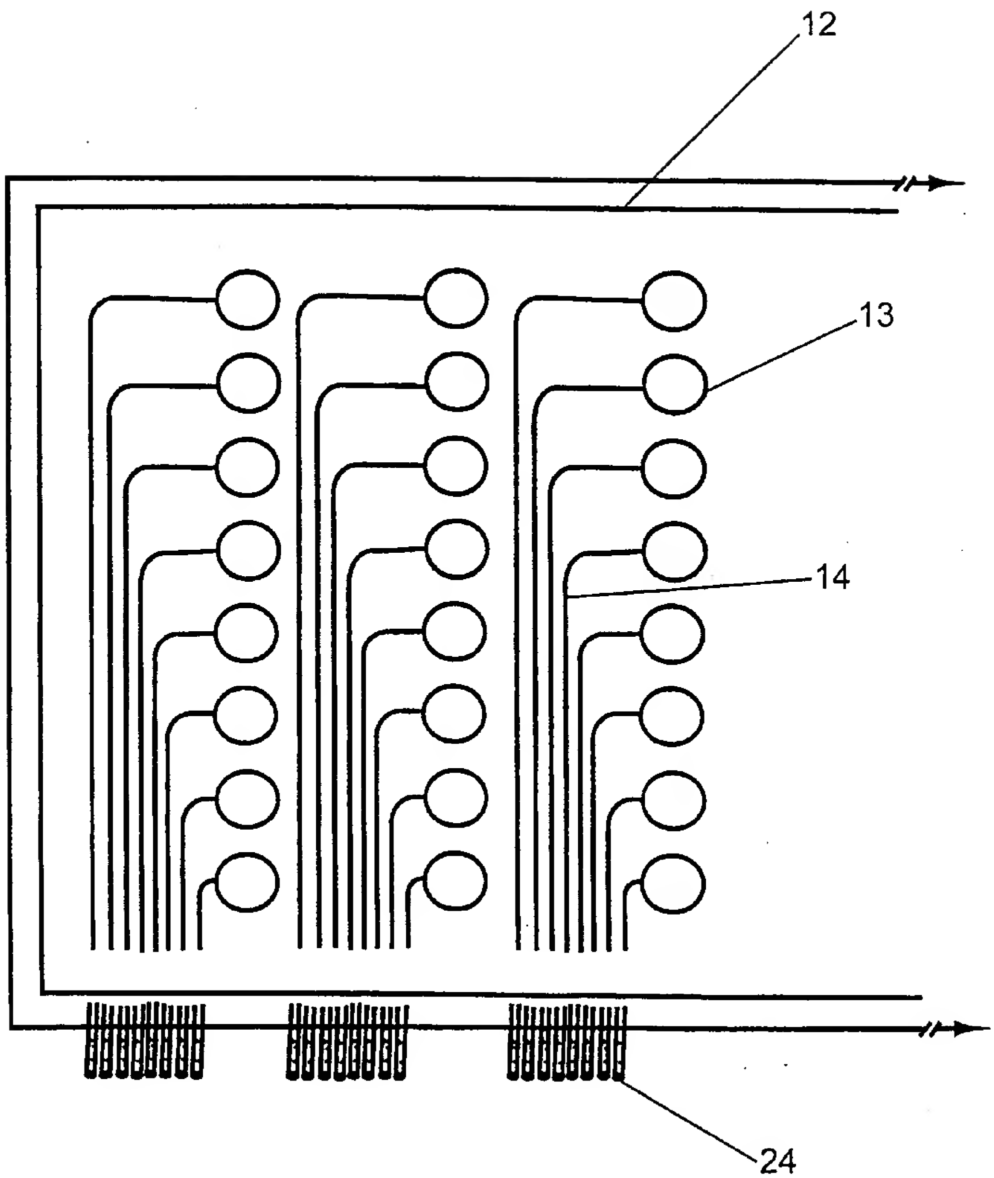


Figure 10

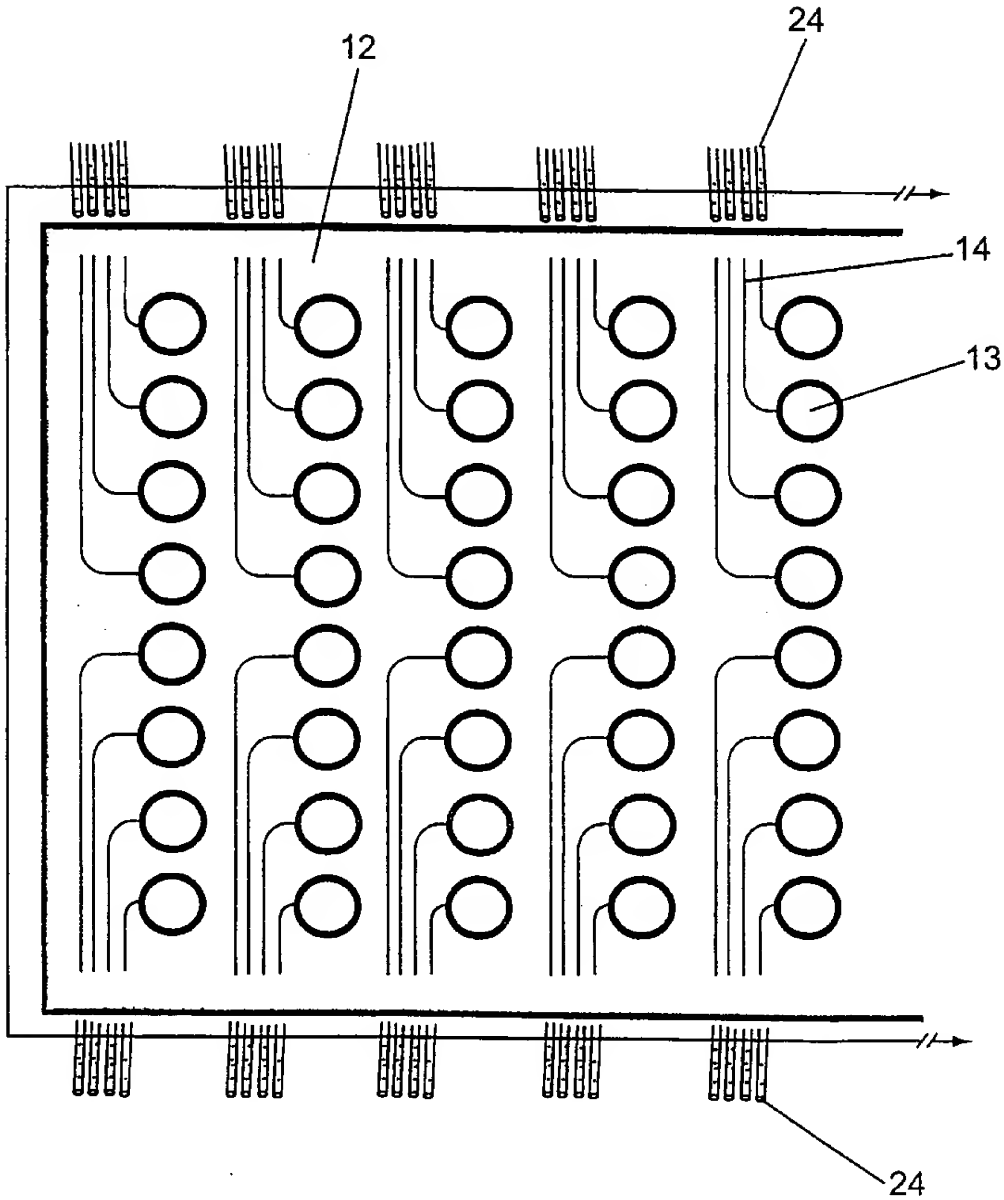


Figure 11

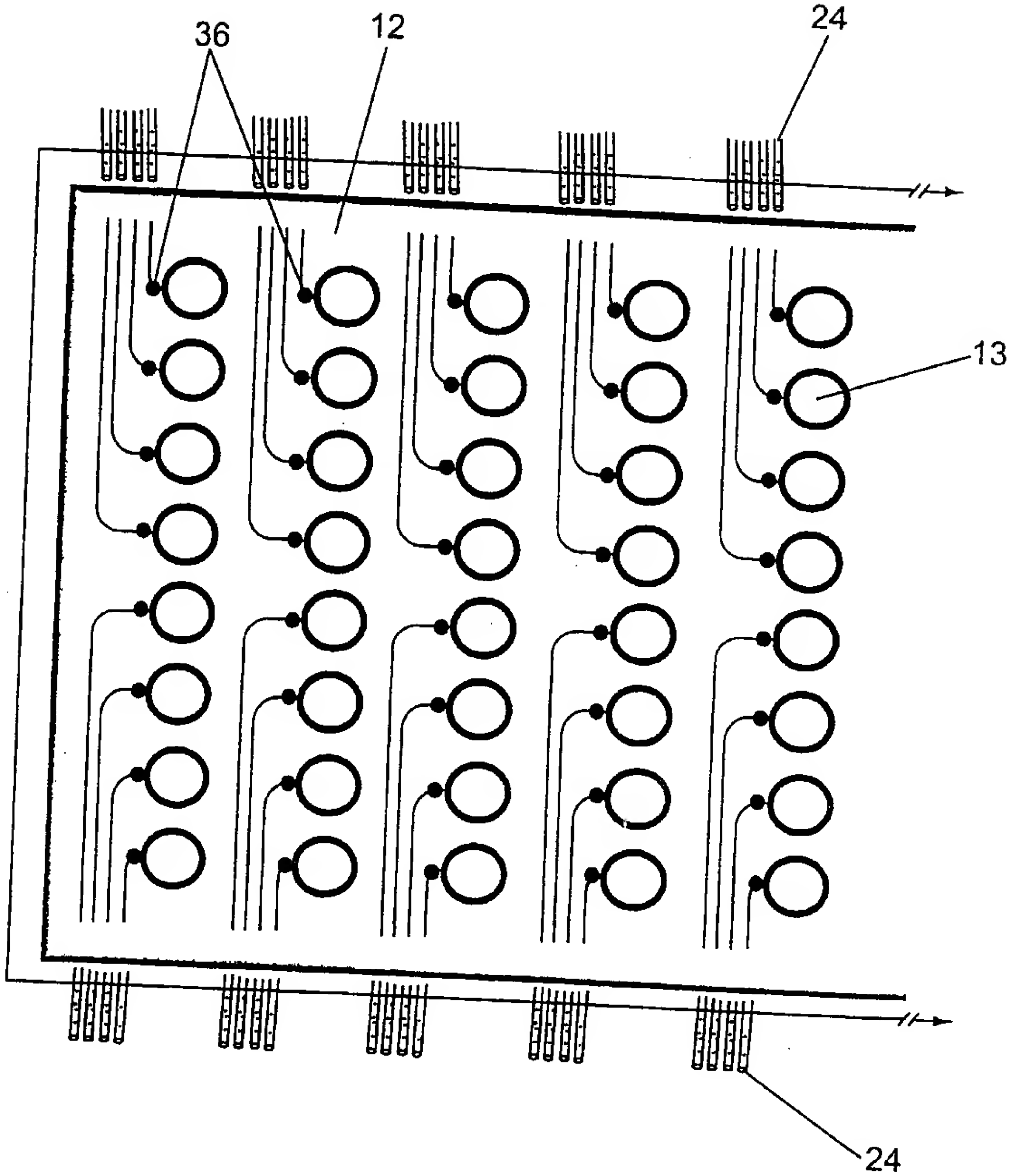


Figure 12

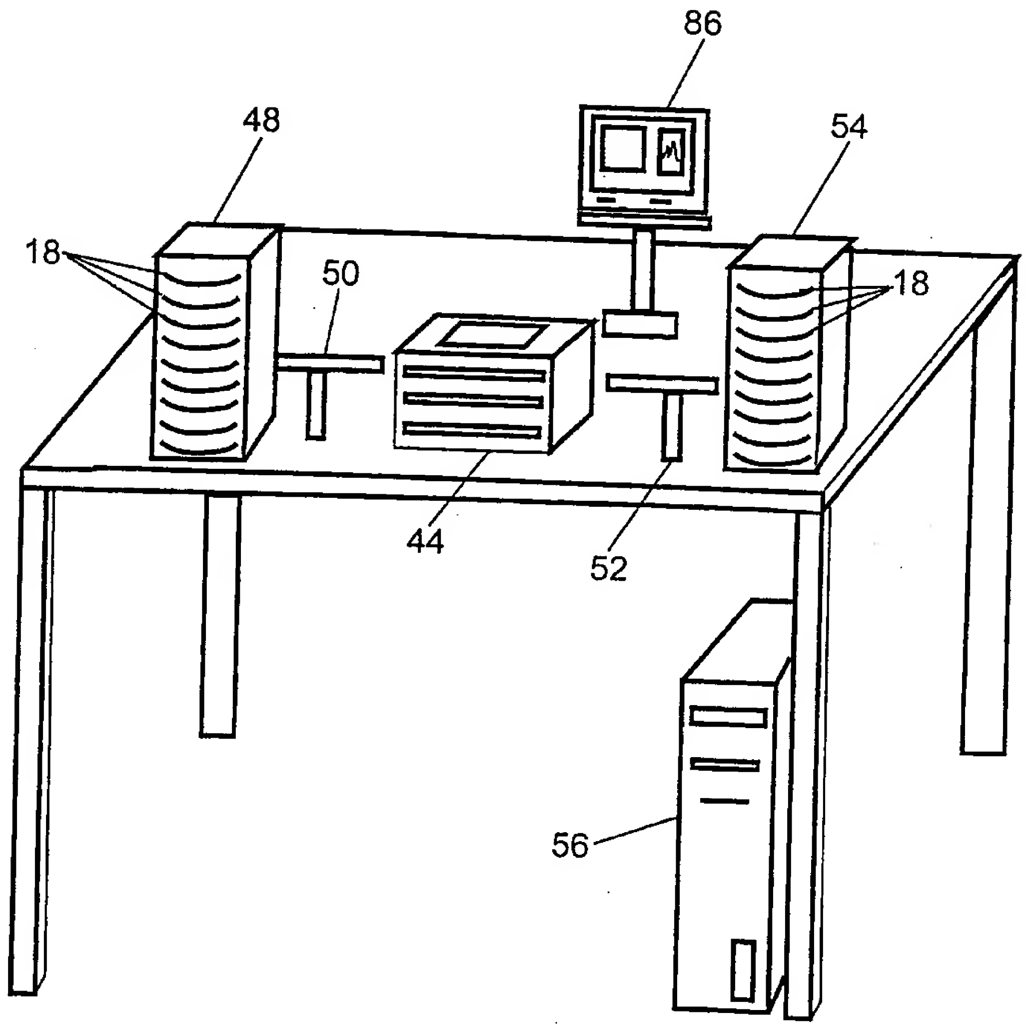


Figure 13

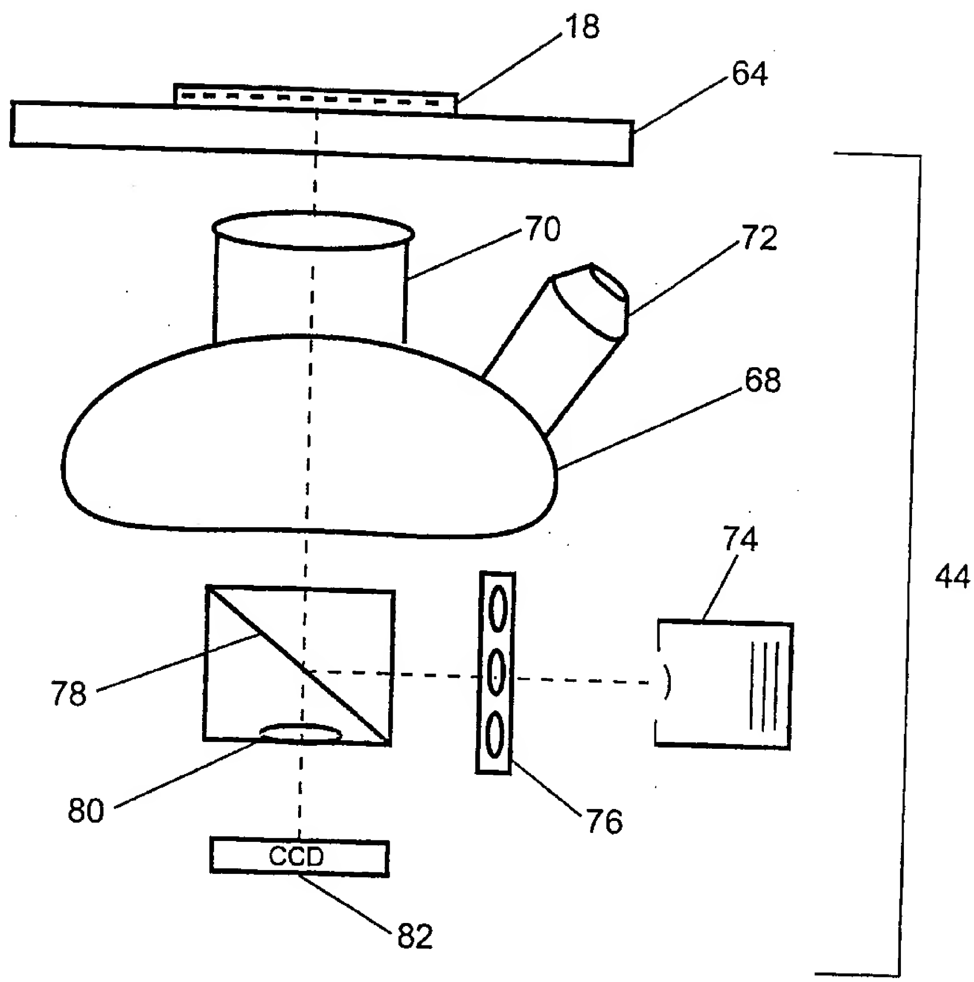


Figure 14A

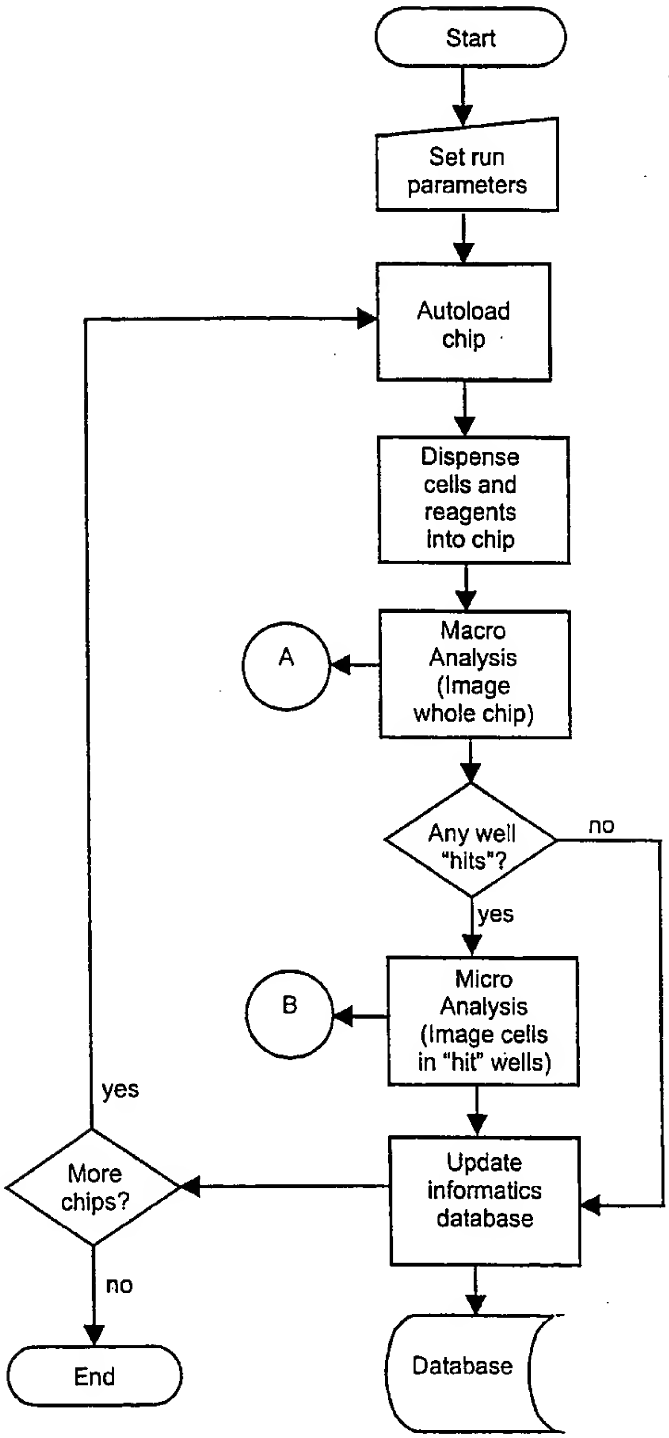

Start
Set run parameters
Autoload chip
Dispense cells and reagents into chip
Macro Analysis (Image whole chip)
A
Any well "hits"?
no
yes
Micro Analysis (Image cells in "hit" wells)
B
Update informatics database
More chips?
yes
no
End
Database

Figure 14B

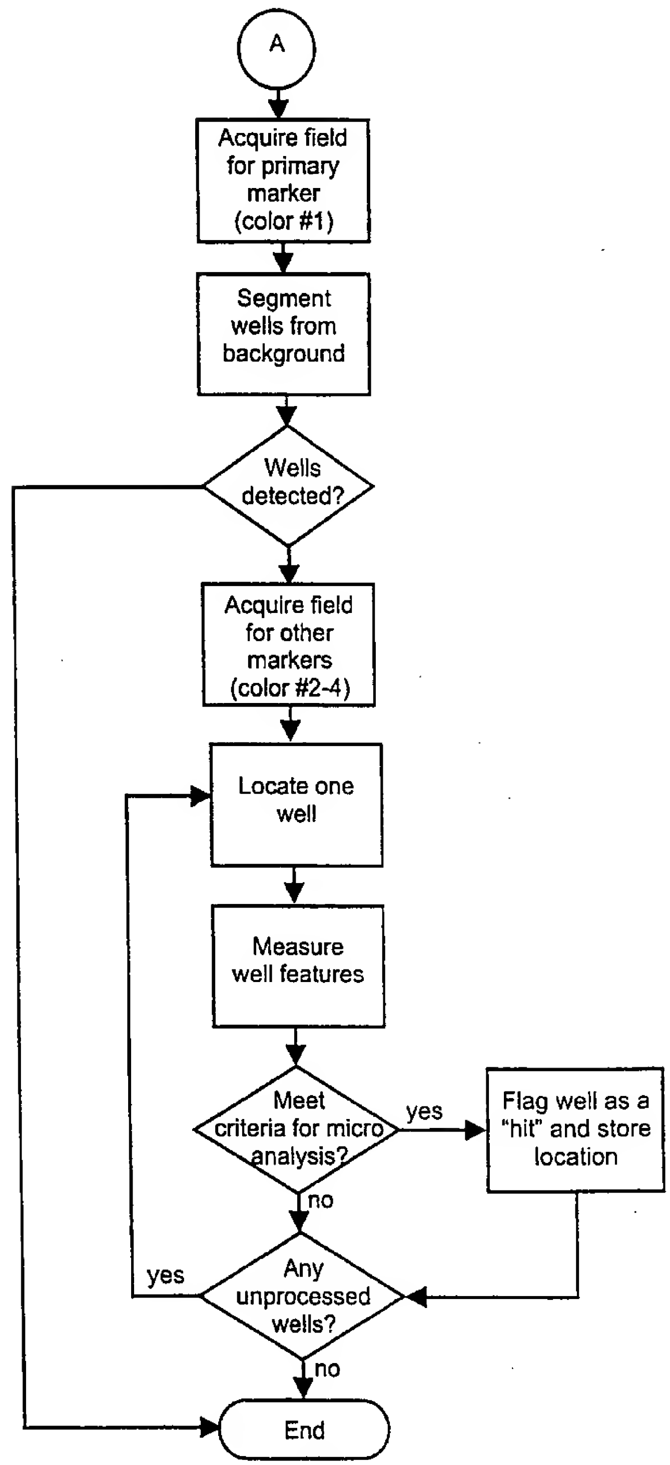

A
Acquire field for primary marker (color #1)
Segment wells from background
Wells detected?
Acquire field for other markers (color #2-4)
Locate one well
Measure well features
Meet criteria for micro analysis?
yes
Flag well as a "hit" and store location
no
yes
Any unprocessed wells?
no
End

Figure 14C

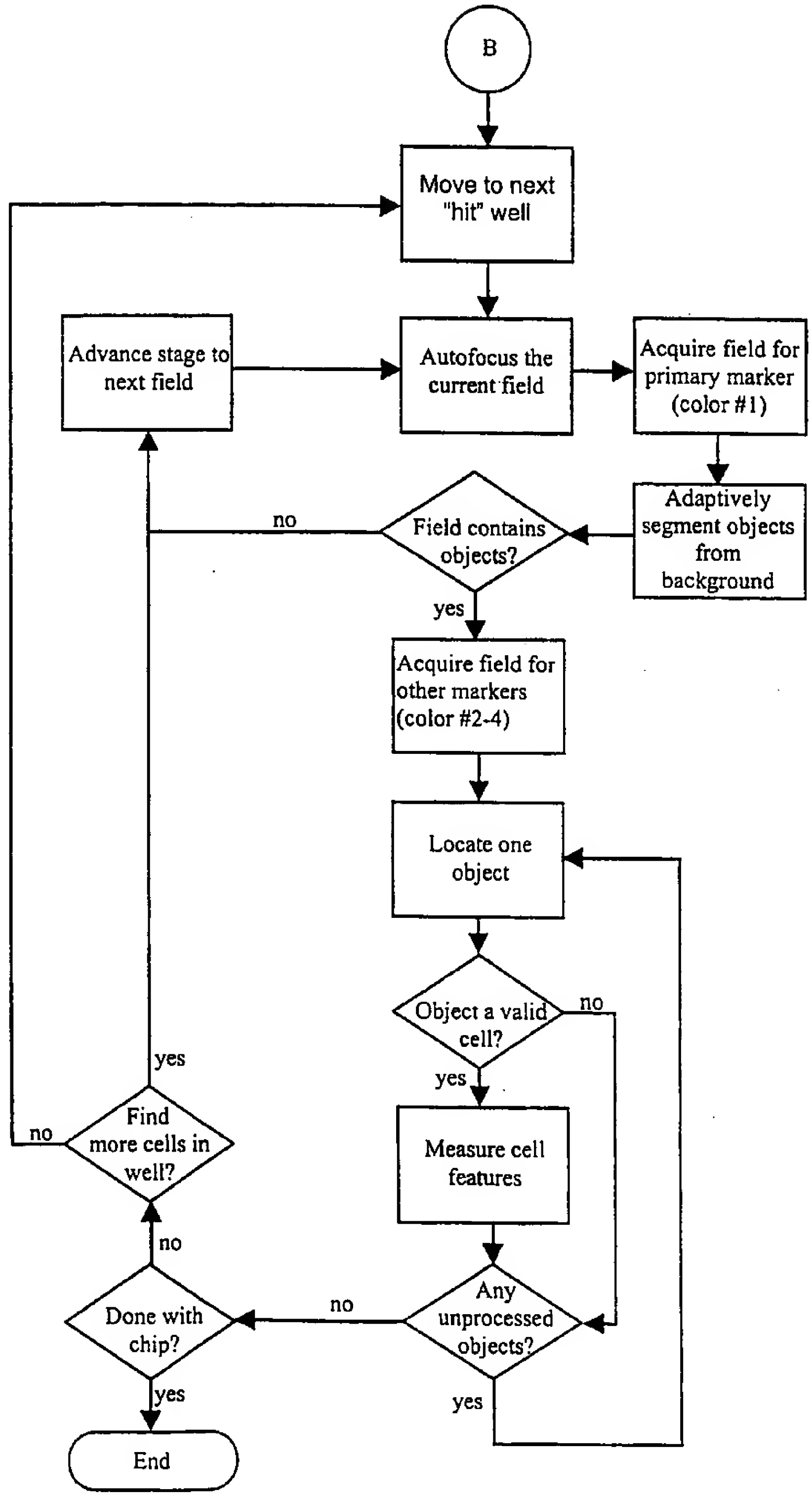

B
Move to next "hit" well
Advance stage to next field
Autofocus the current field
Acquire field for primary marker (color #1)
Adaptively segment objects from background
Field contains objects?
no
yes
Acquire field for other markers (color #2-4)
Locate one object
Object a valid cell?
no
yes
Measure cell features
Any unprocessed objects?
yes
no
Find more cells in well?
yes
no
Done with chip?
no
yes
End

Figure 15

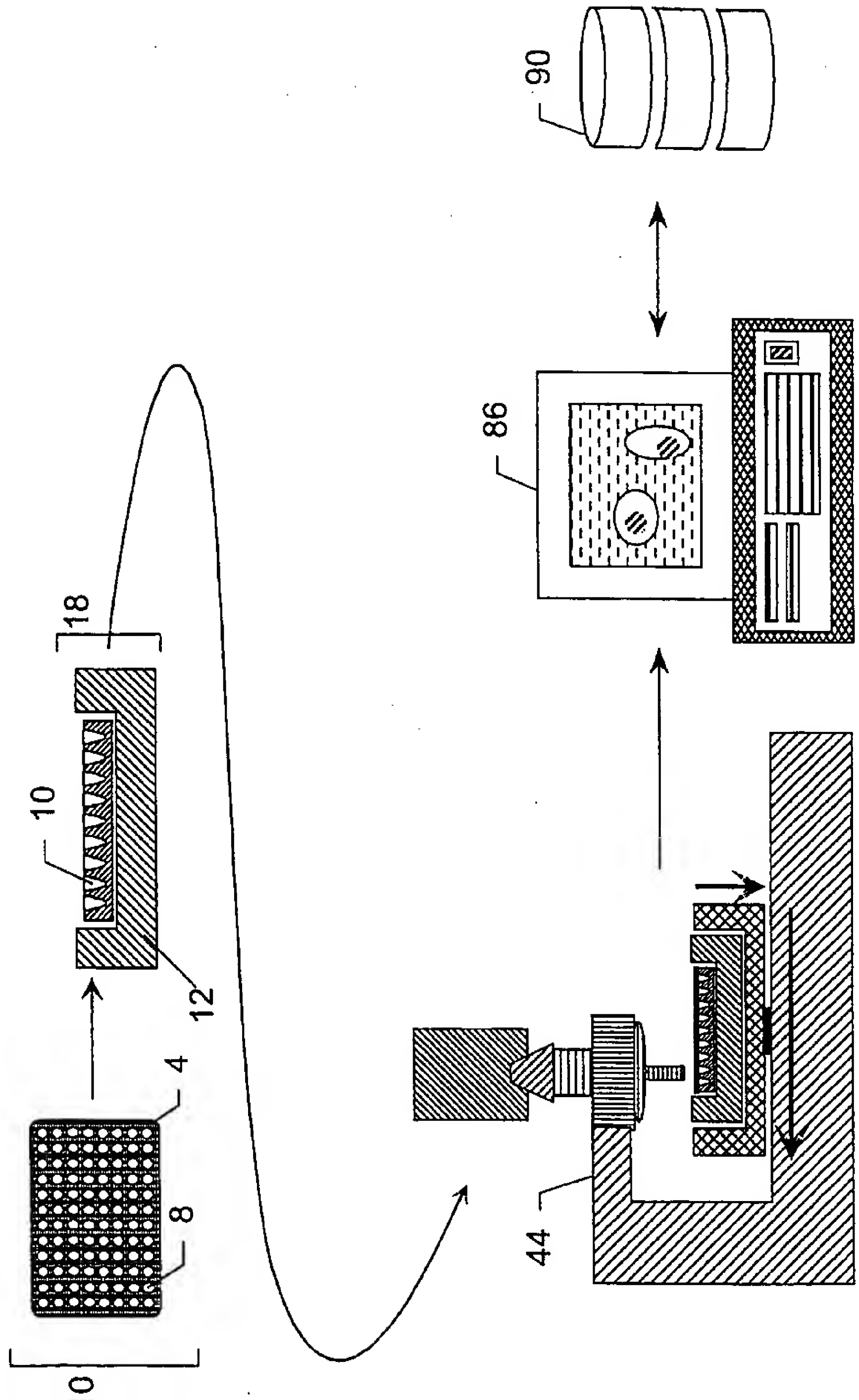


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Figure 16

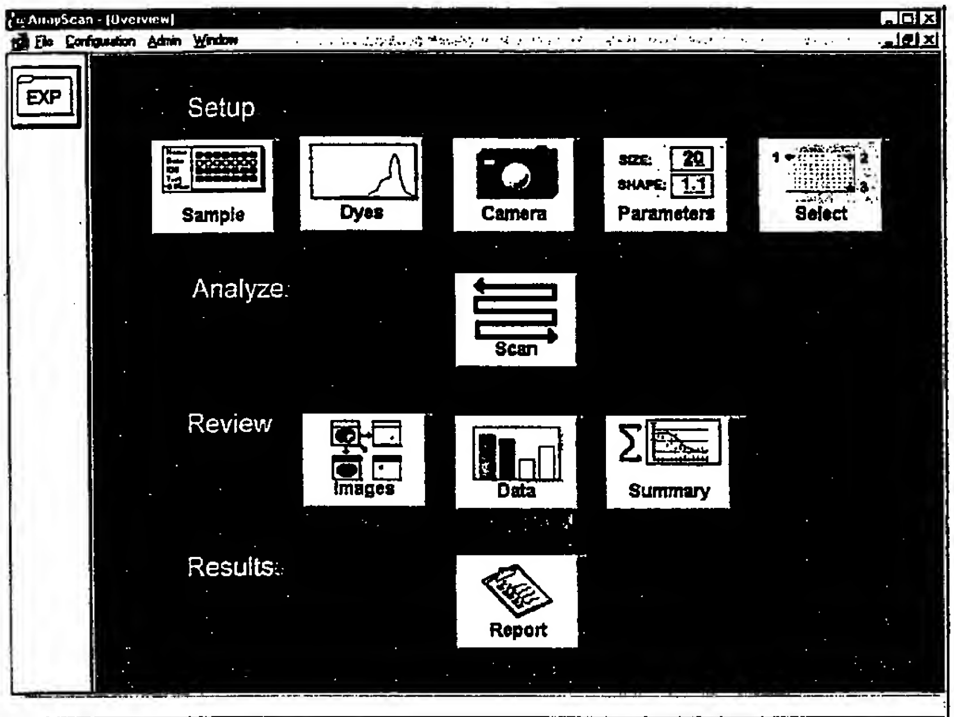

EXP
Setup
Sample
Dyes
Camera
SIZE: 20
SHAPE: 1.1
Parameters
Select
Analyze:
Scan
Review
Images
Data
Summary
Results:
Report

Figure 17

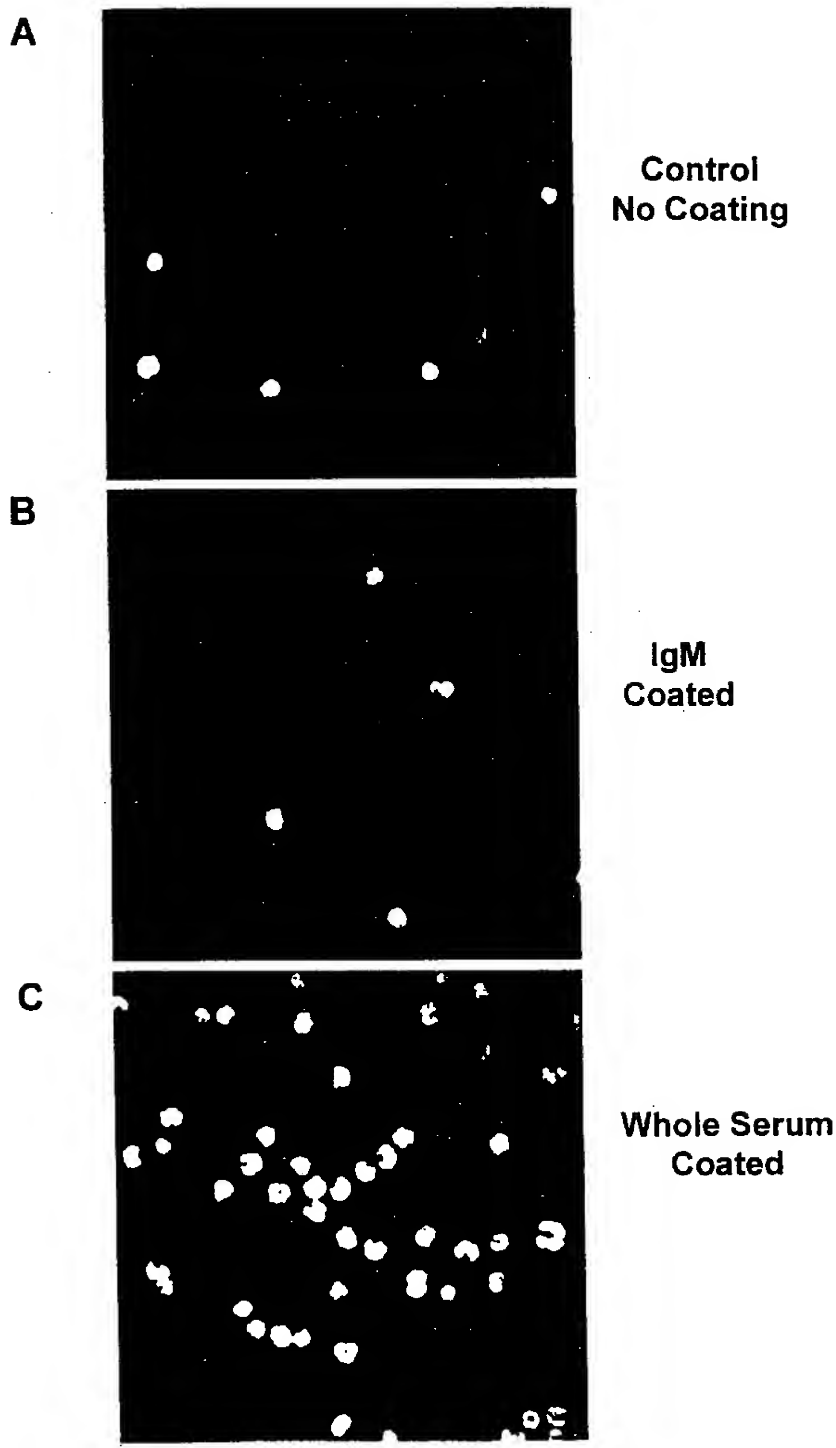
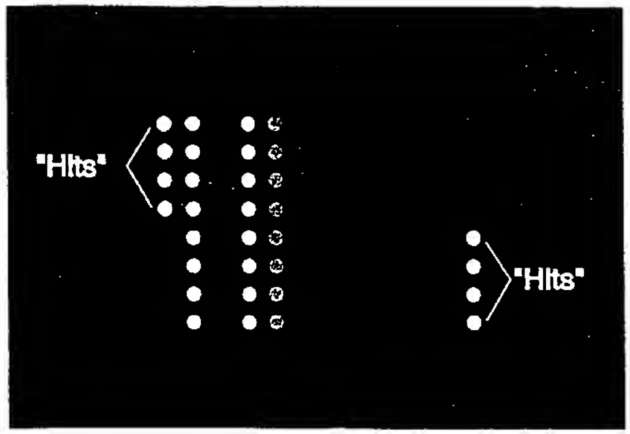


Figure 18

A



B

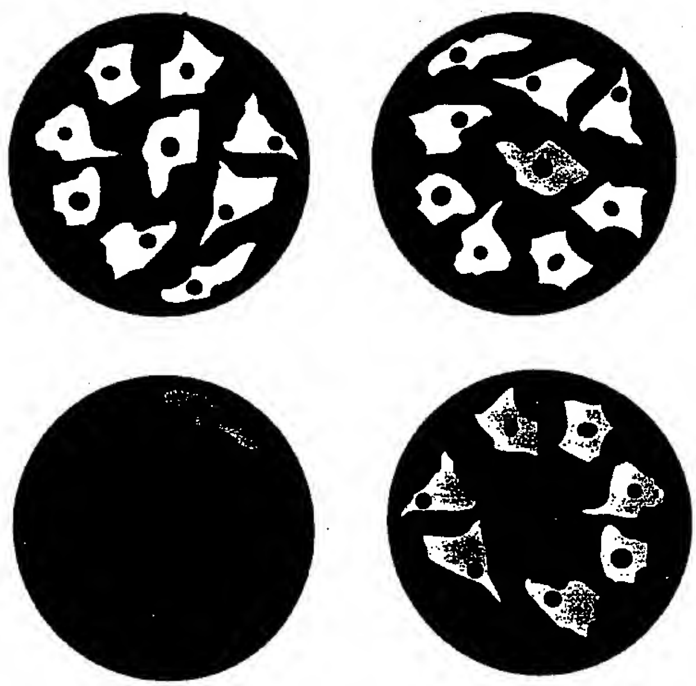
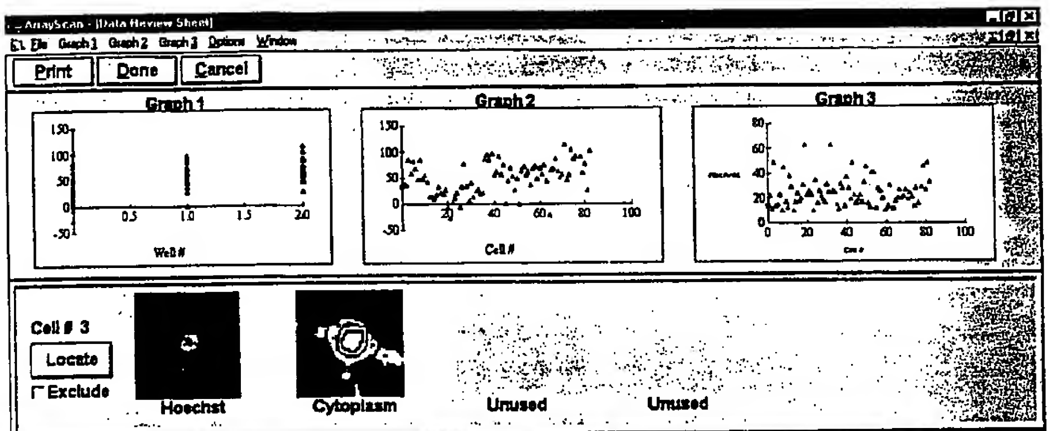


Figure 19



	Cell #	Well #	Field #	Cell # in Well	NucSum	NucArea	NucAvg	NP1Inten	NP1Avg	NucL1P1AvgRatio	L1P1Inten	L1P1Avg	L1P1Area	NucL1P1AvgDiff
1	0	A1	1	0	1634	14	117	2197	157	1.26	10989	125	88	32
2	1	A1	1	1	2897	22	132	3800	173	1.29	12564	134	94	39
3	2	A1	1	2	1654	11	150	1889	172	1.27	11039	135	82	37
4	3	A1	1	3	7293	49	149	10531	215	1.65	14003	130	108	85
5	4	A1	1	4	1947	14	139	2603	186	1.47	10918	127	86	59
6	5	A1	1	5	1600	15	107	3571	238	1.53	13404	156	86	82
7	6	A1	1	6	3866	23	168	4565	198	1.52	12995	130	100	68
8	7	A1	1	7	1459	10	146	1837	184	1.34	11545	137	84	47
9	8	A1	1	8	7038	45	156	9872	219	1.63	13955	134	104	85
10	9	A1	1	9	2070	17	122	2672	157	1.45	9499	108	88	49
11	10	A1	1	10	1748	13	134	2051	158	1.53	8651	103	84	55
12	11	A1	1	11	6829	38	180	6295	166	1.35	12272	123	100	43
13	12	A1	1	12	4501	29	155	3406	117	1.13	10167	104	98	13
14	13	A1	1	13	1361	10	136	897	89	1.19	6522	75	87	14
15	14	A1	1	14	2351	17	138	1666	98	1.11	7921	88	90	10
16	15	A1	1	15	3327	24	139	3277	137	1.14	11001	120	92	17
17	16	A1	1	16	3021	18	168	2542	141	1.31	9729	100	90	33
18	17	A1	1	17	2561	20	128	1881	94	1.31	6884	72	96	22

Figure 20

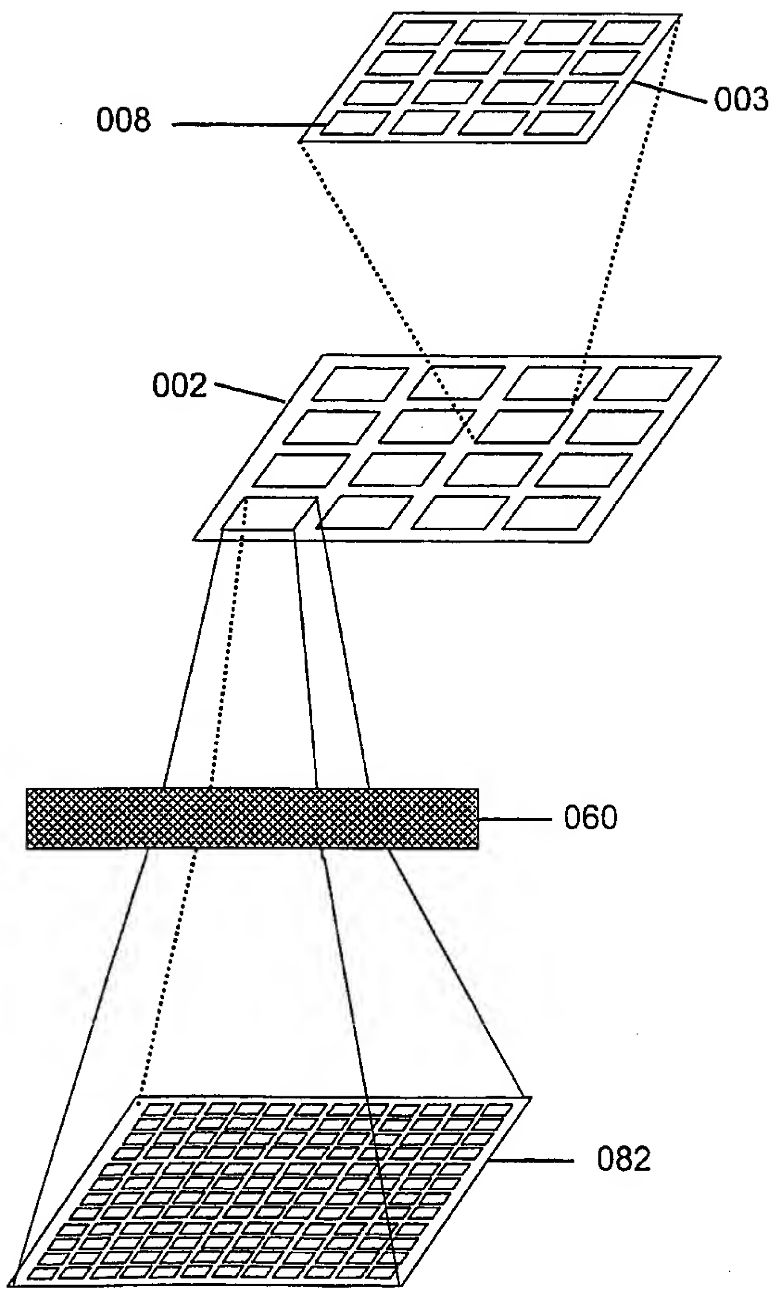


Figure 21

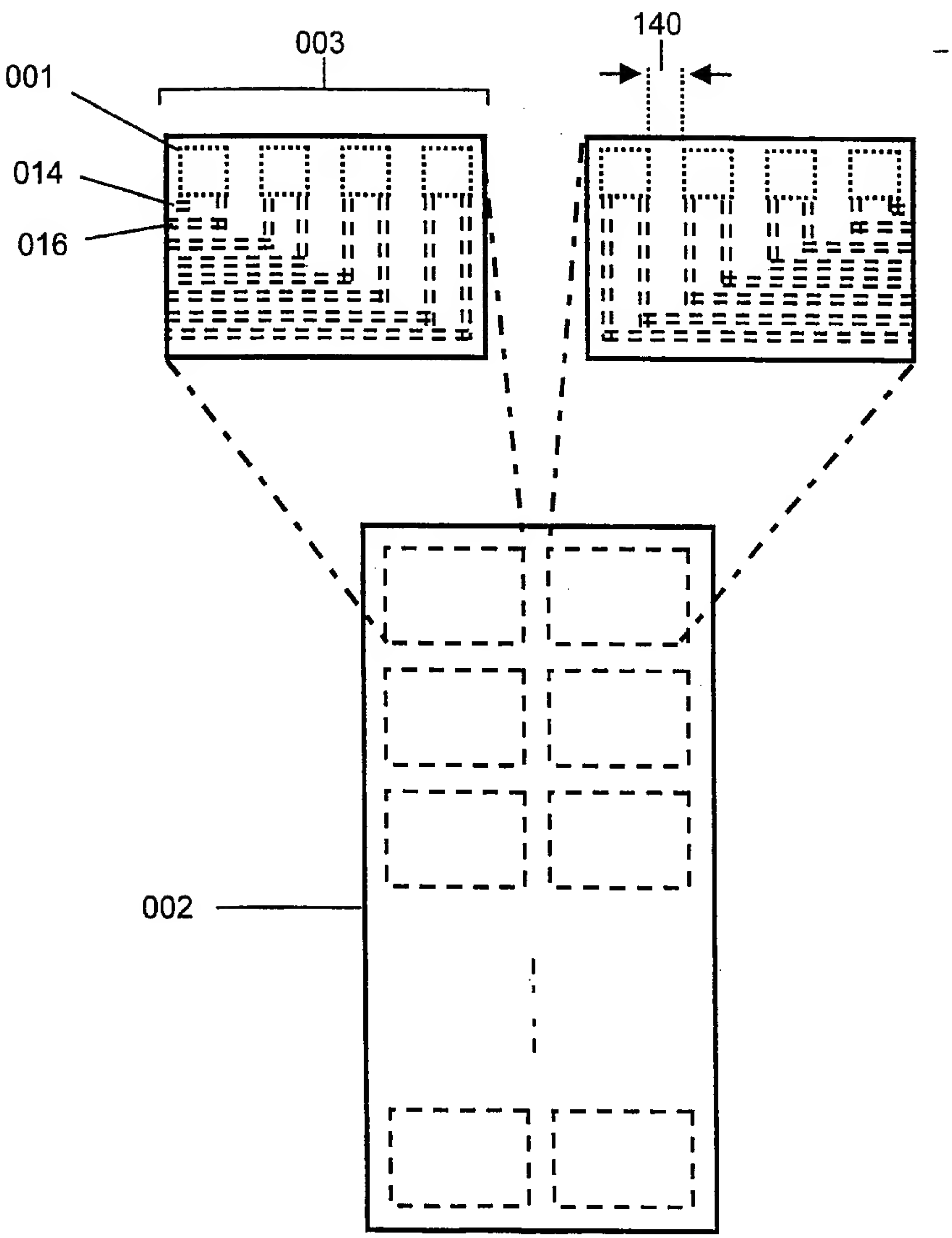


Figure 22

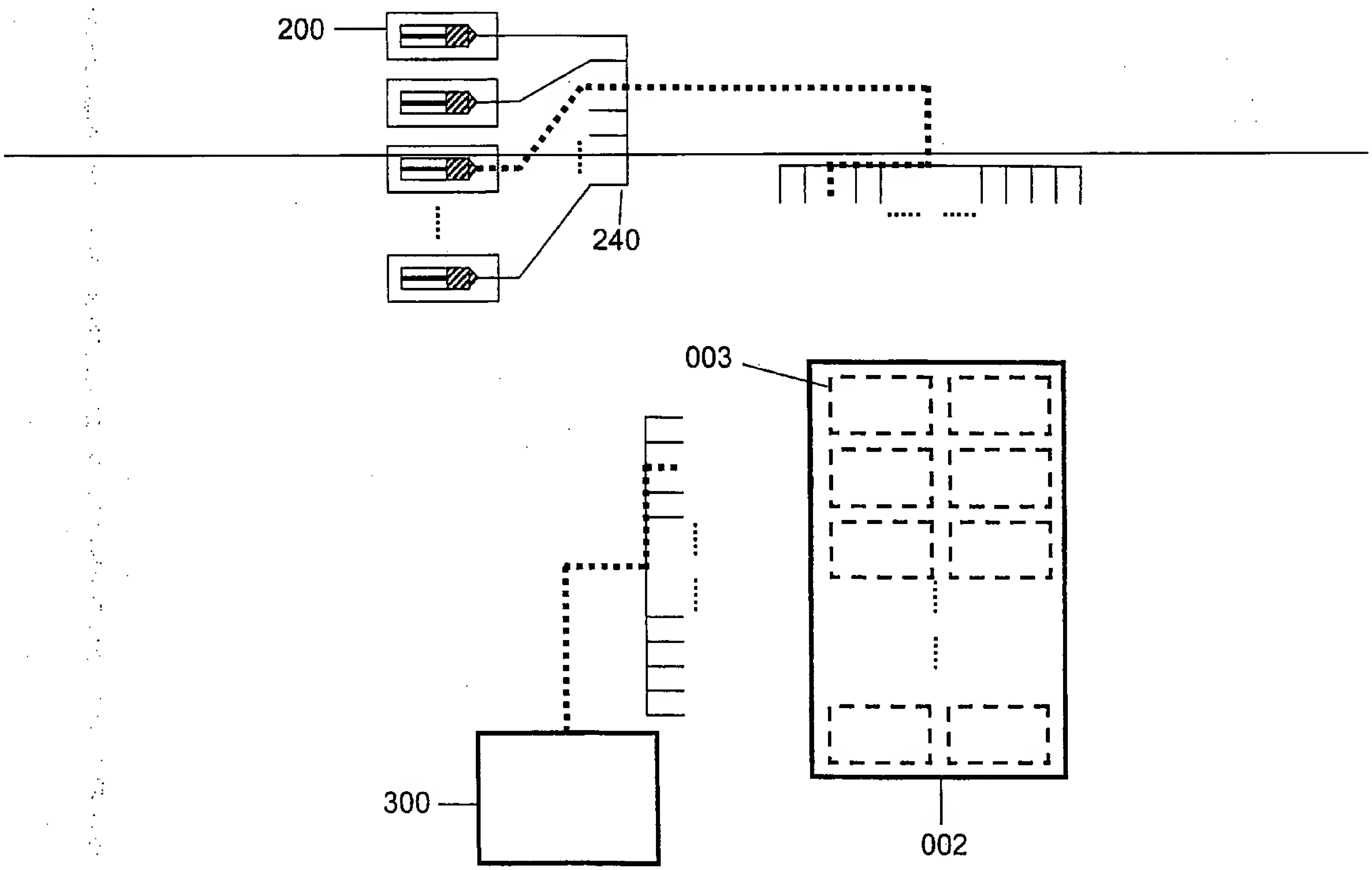


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Figure 23

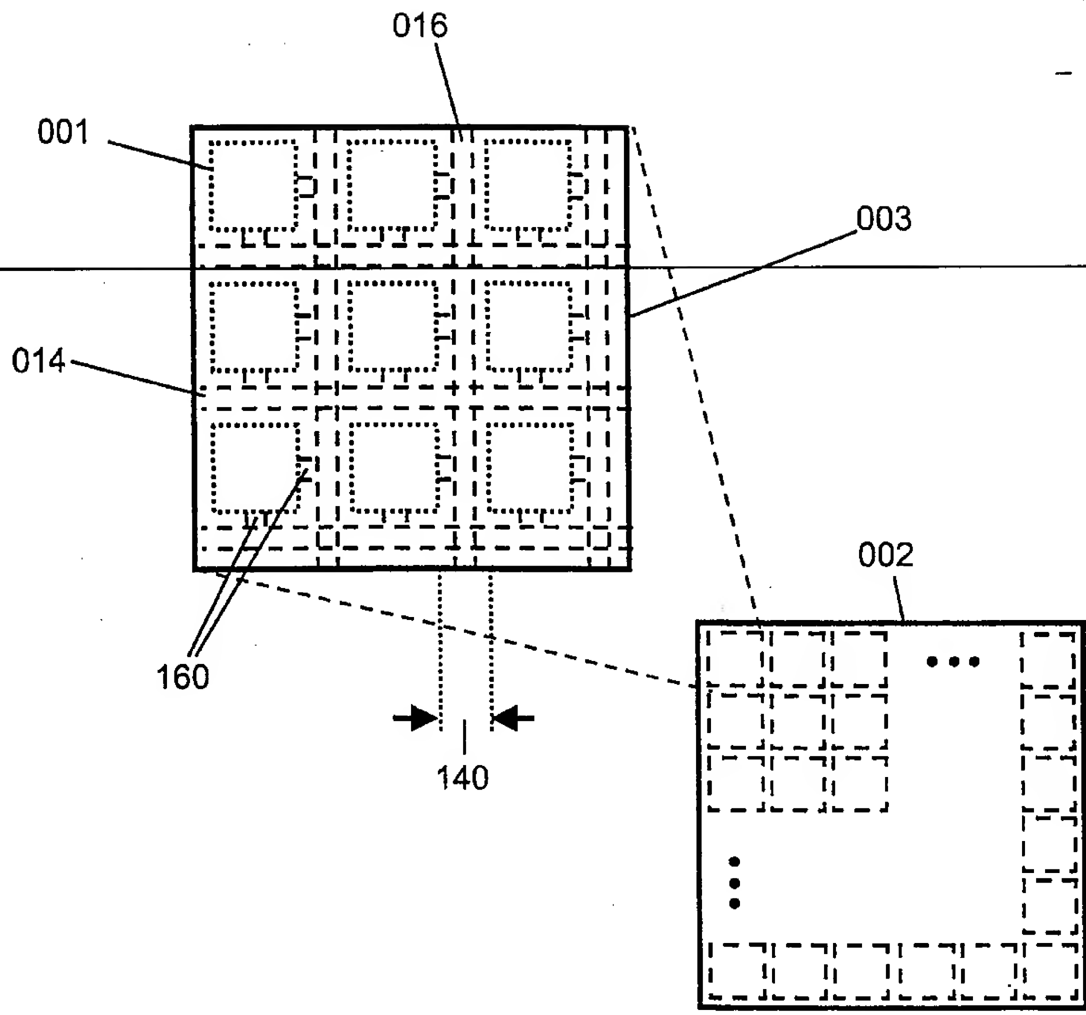


Figure 24

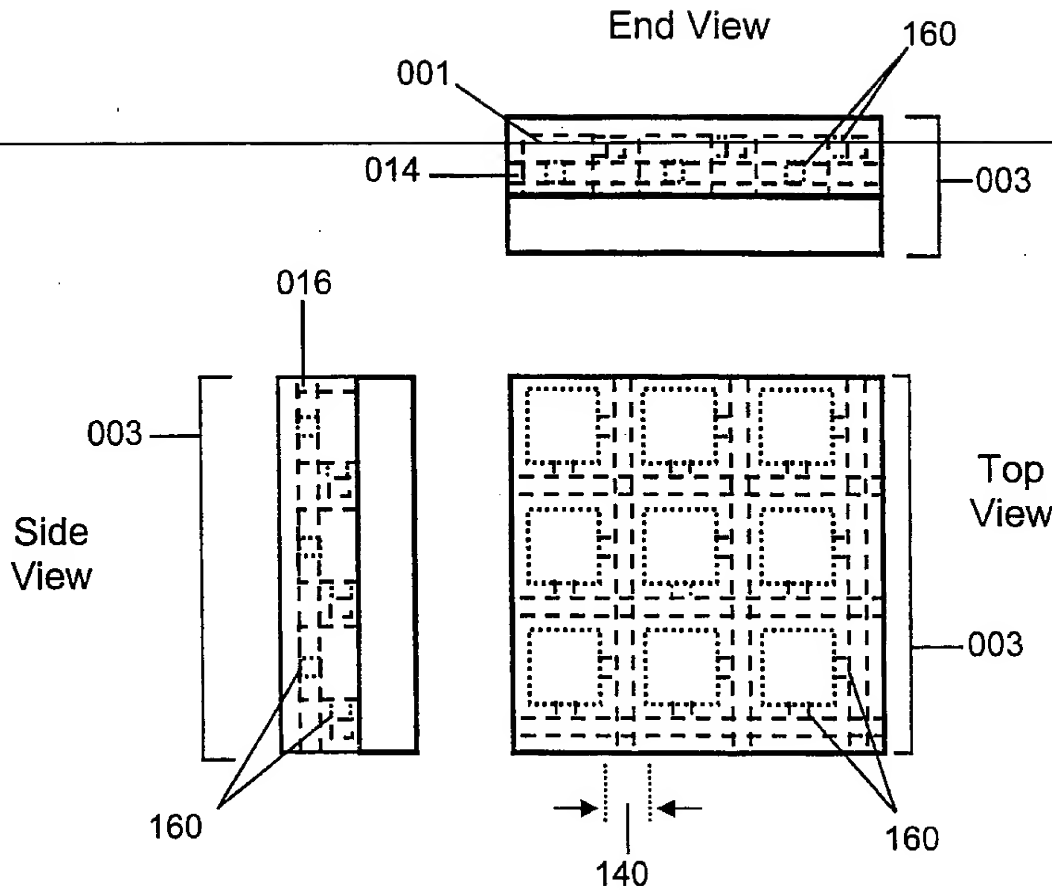

End View
001
160
014
003
016
003
Side View
Top View
003
160
140
160

Figure 25

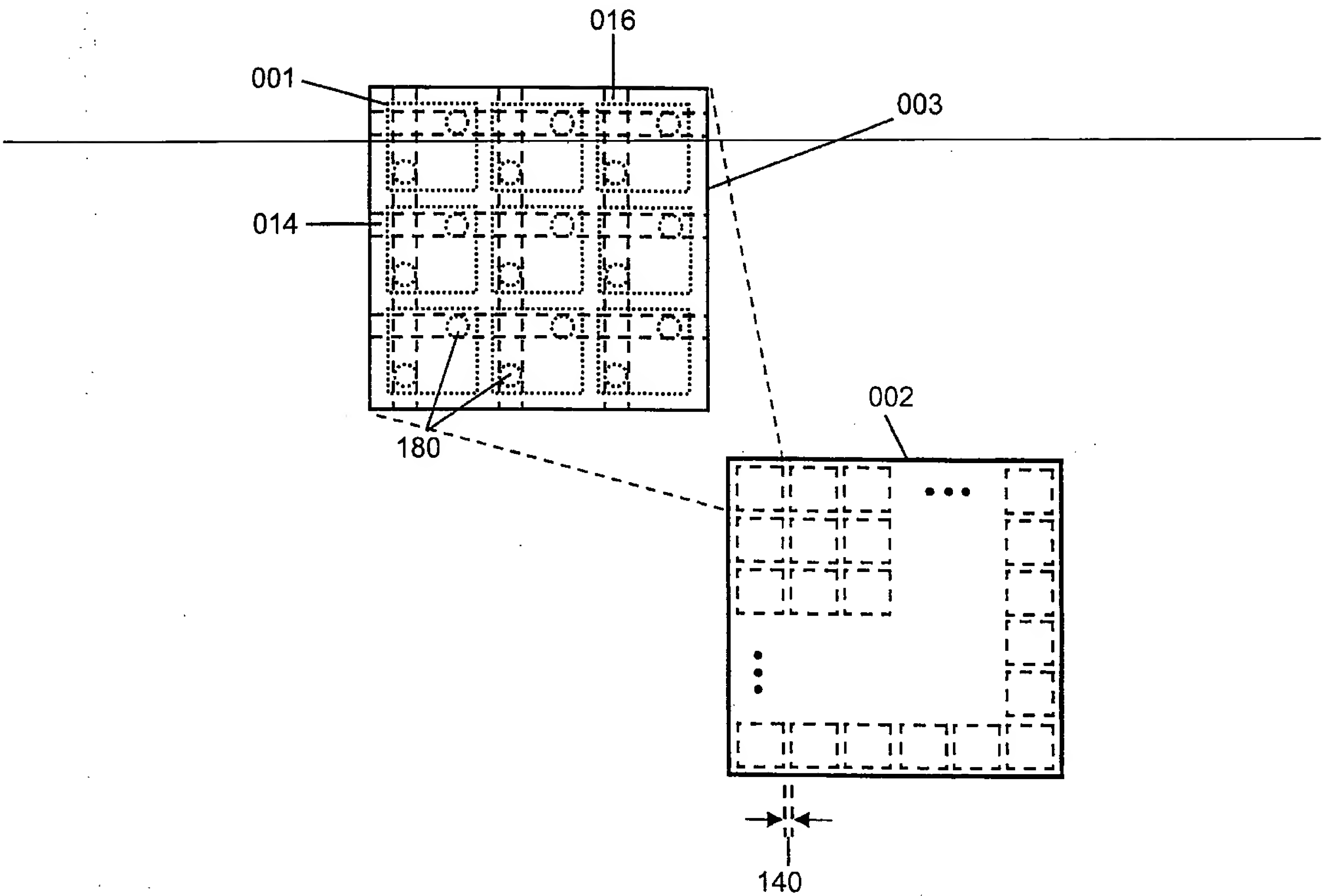
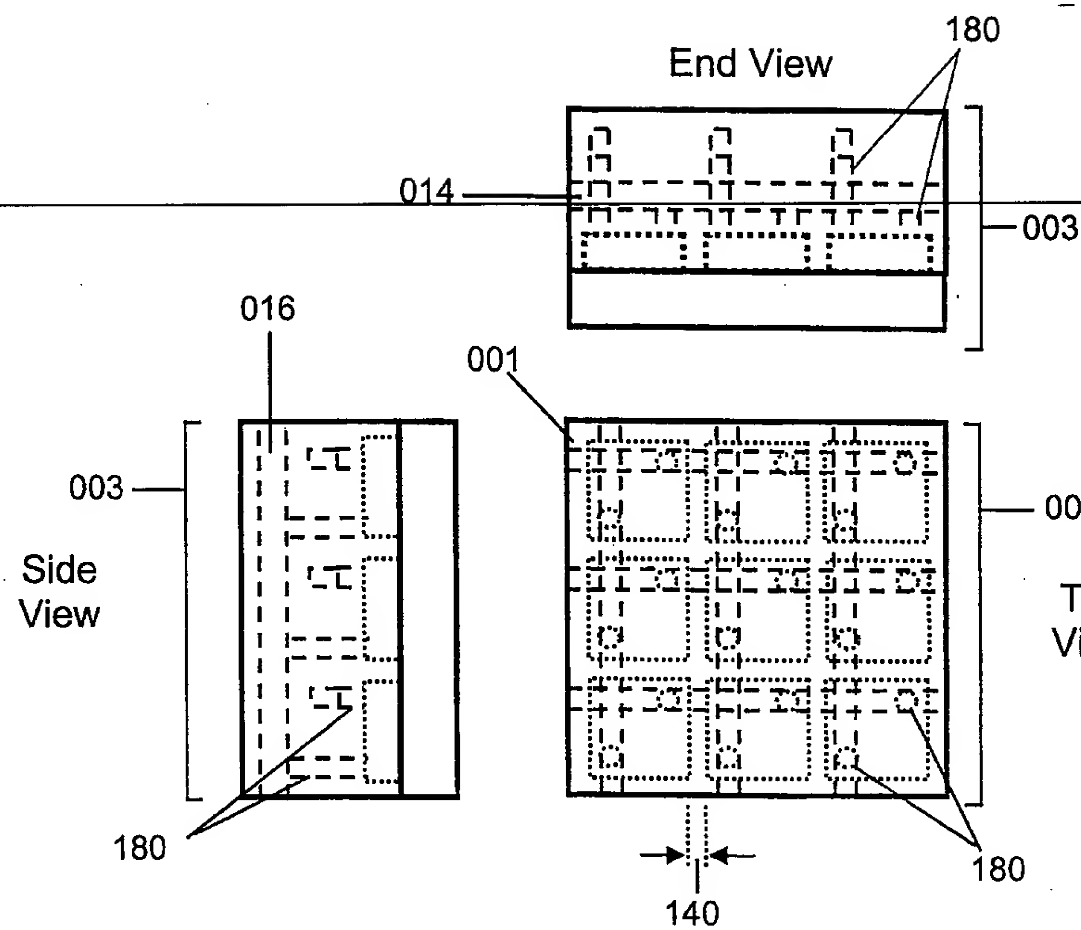


Figure 26



End View
180
014
003
016
001
003
Side
View
003
Top
View
180
140
180

Figure 27

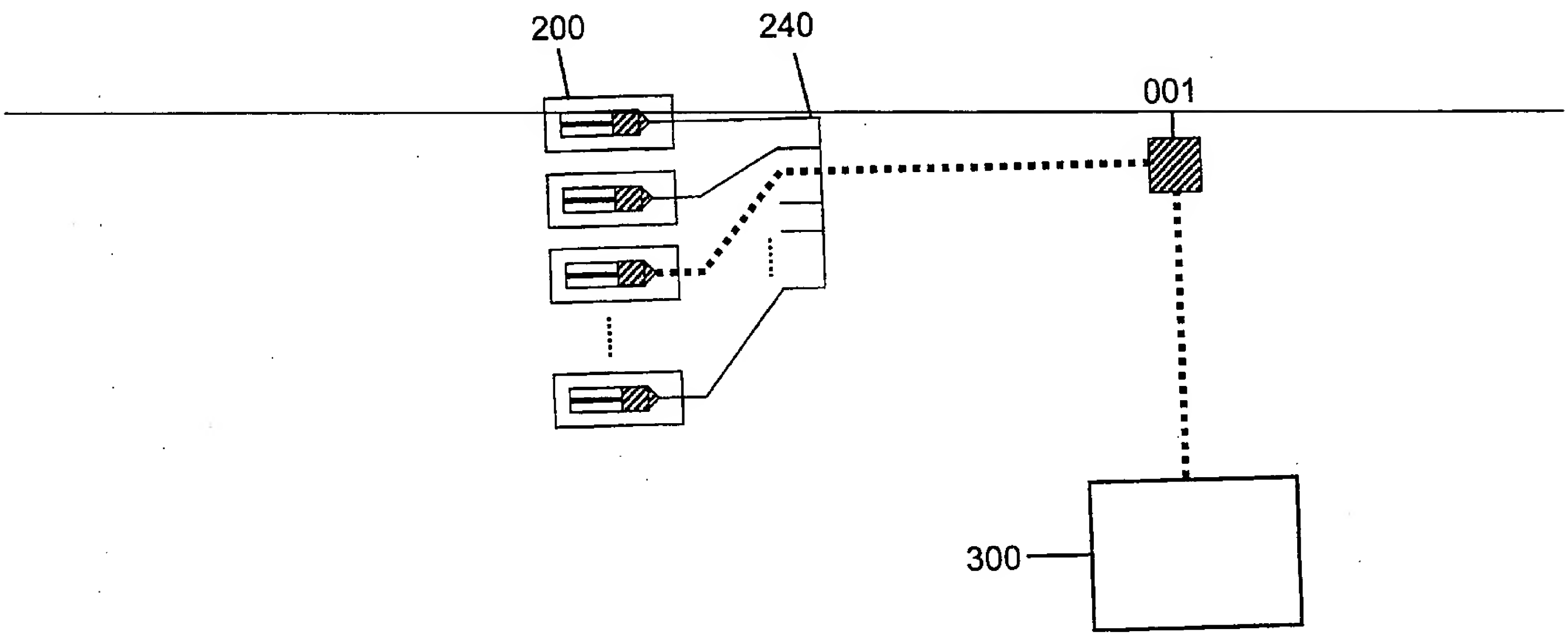


Figure 28

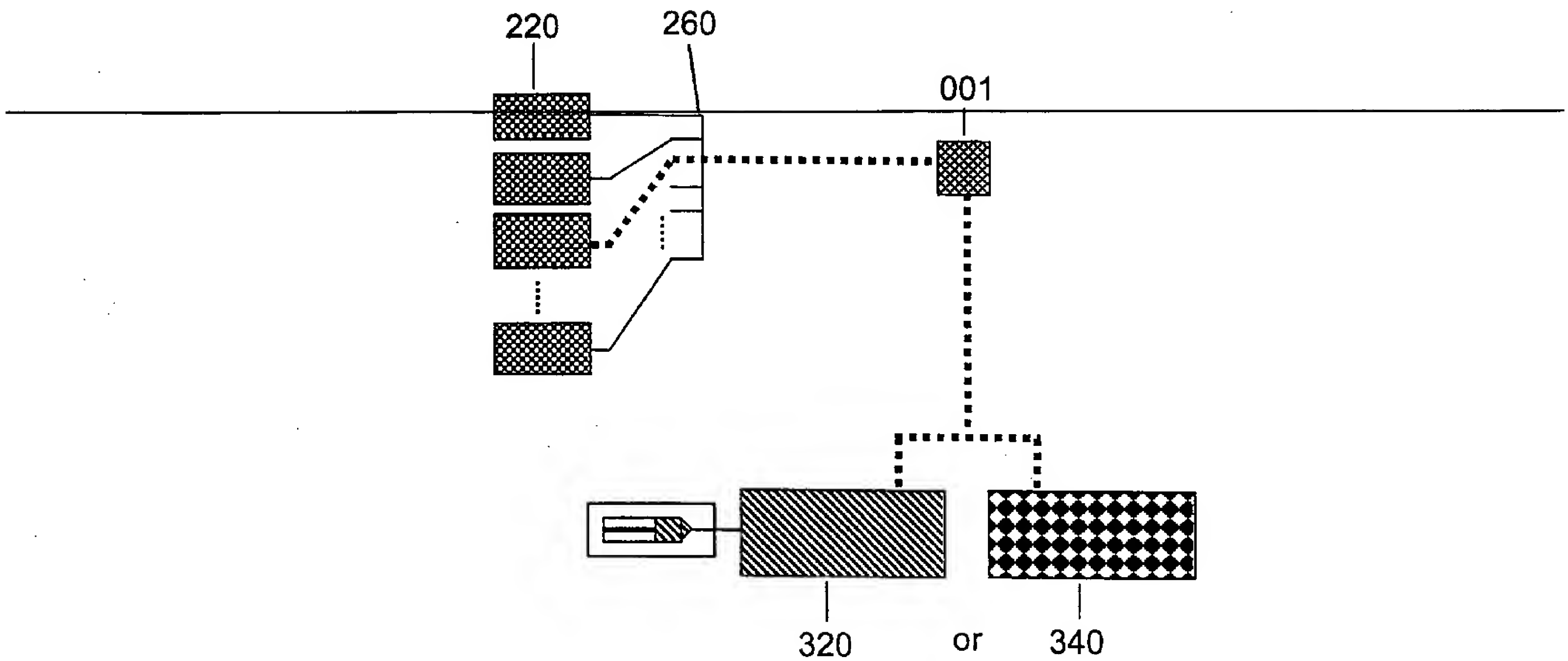


Figure 29

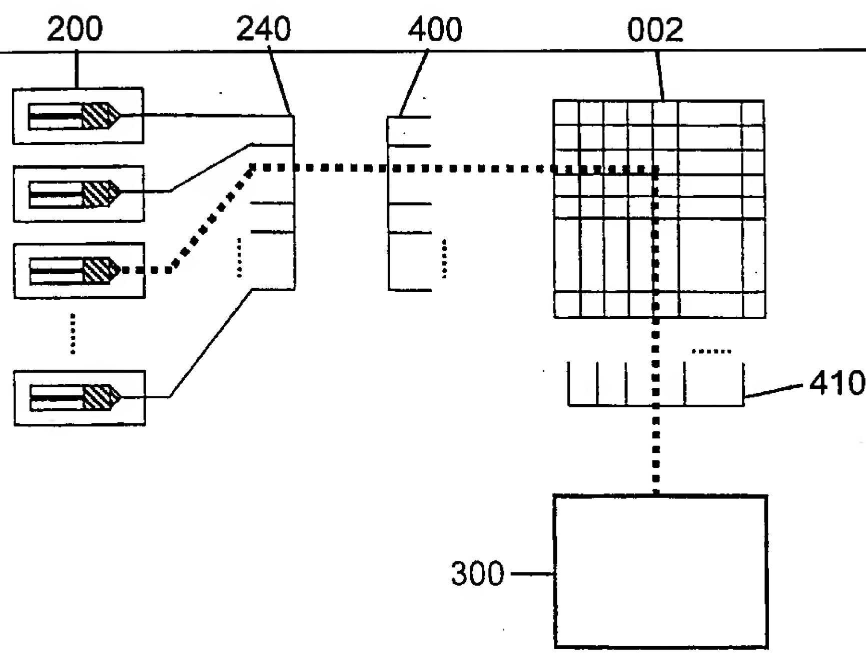


Figure 30

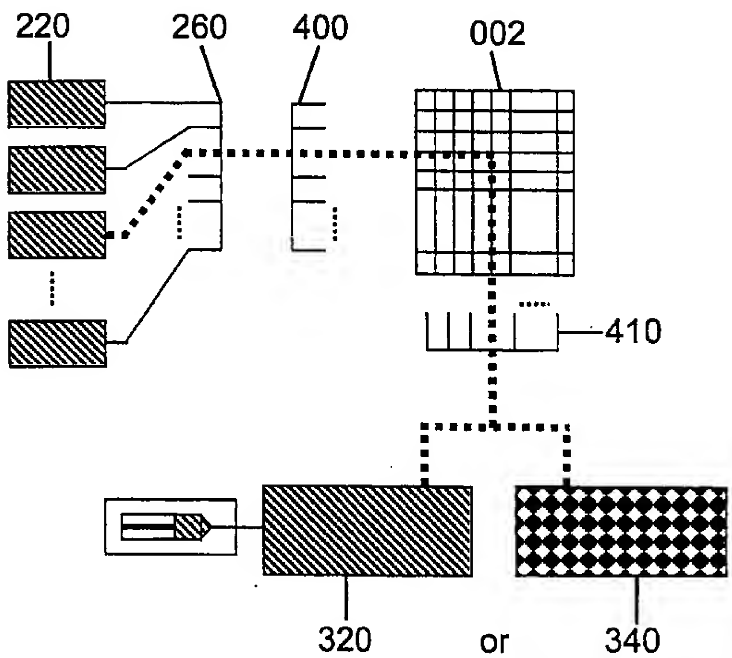


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Figure 31

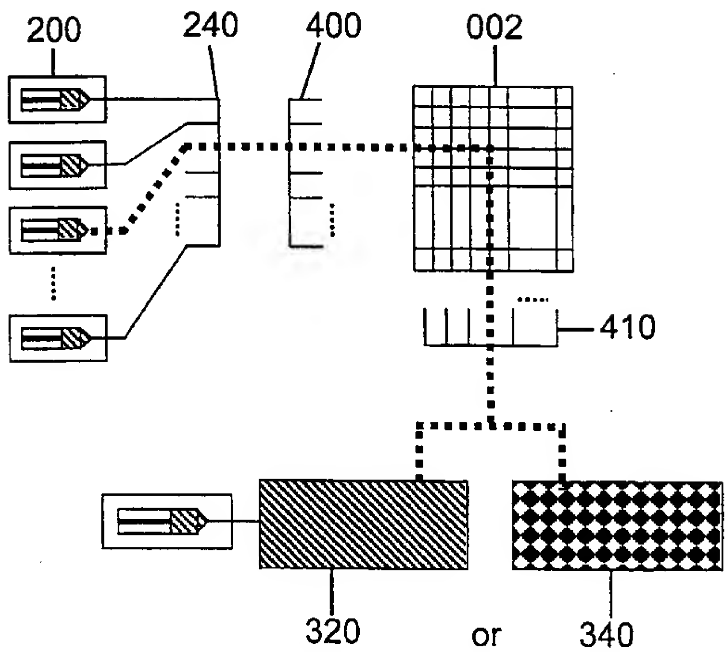

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Figure 32

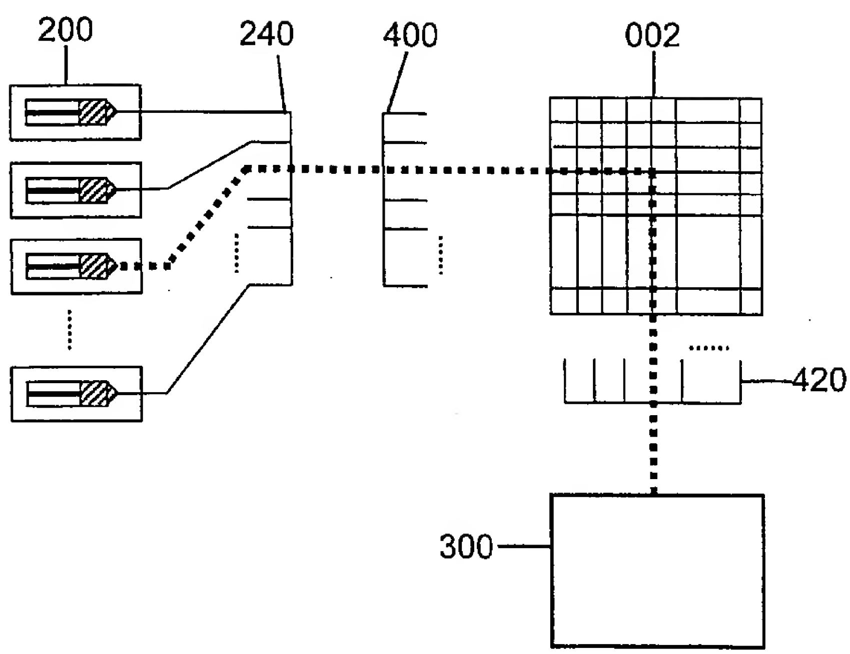

Figure 33

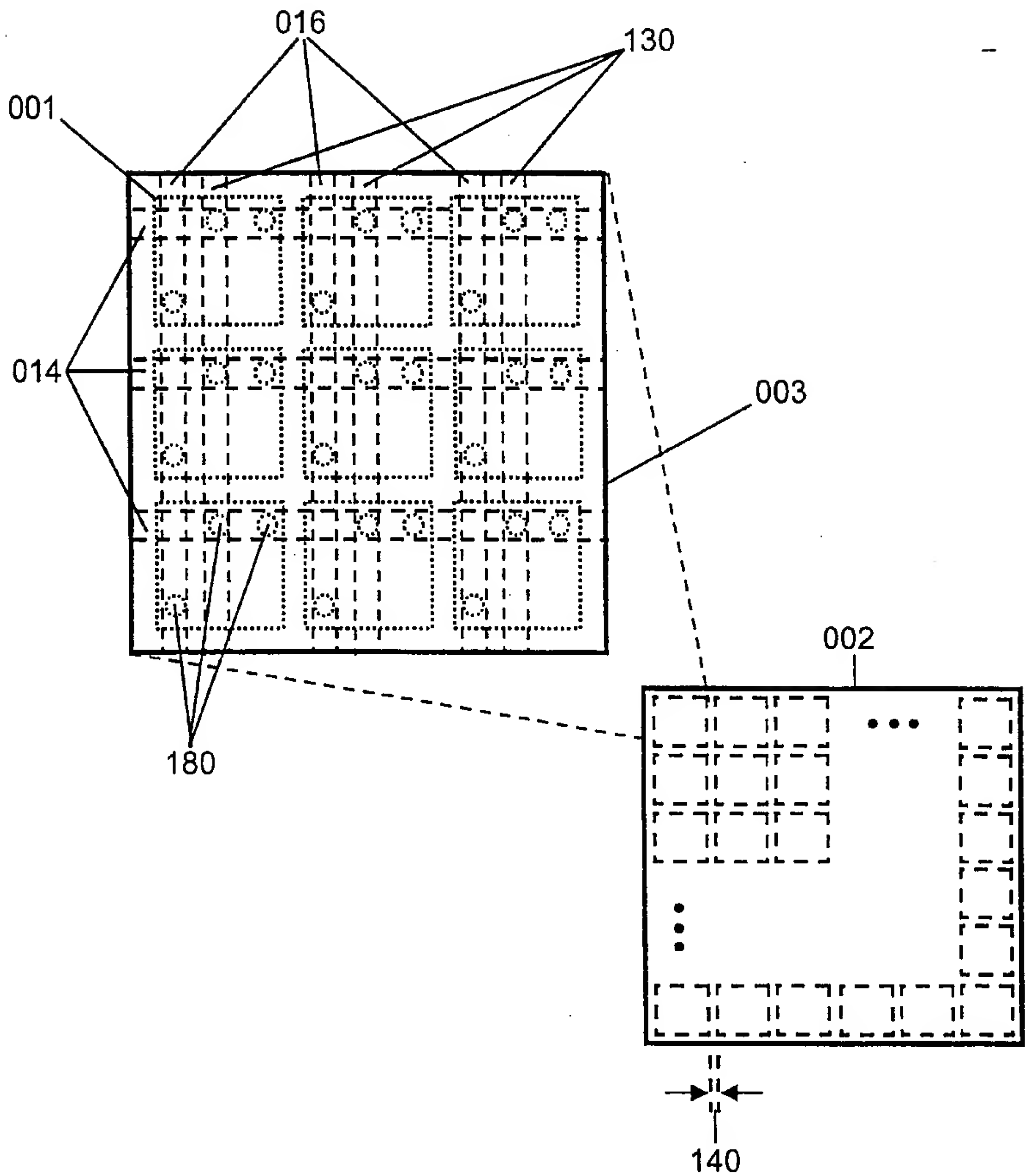

Figure 34

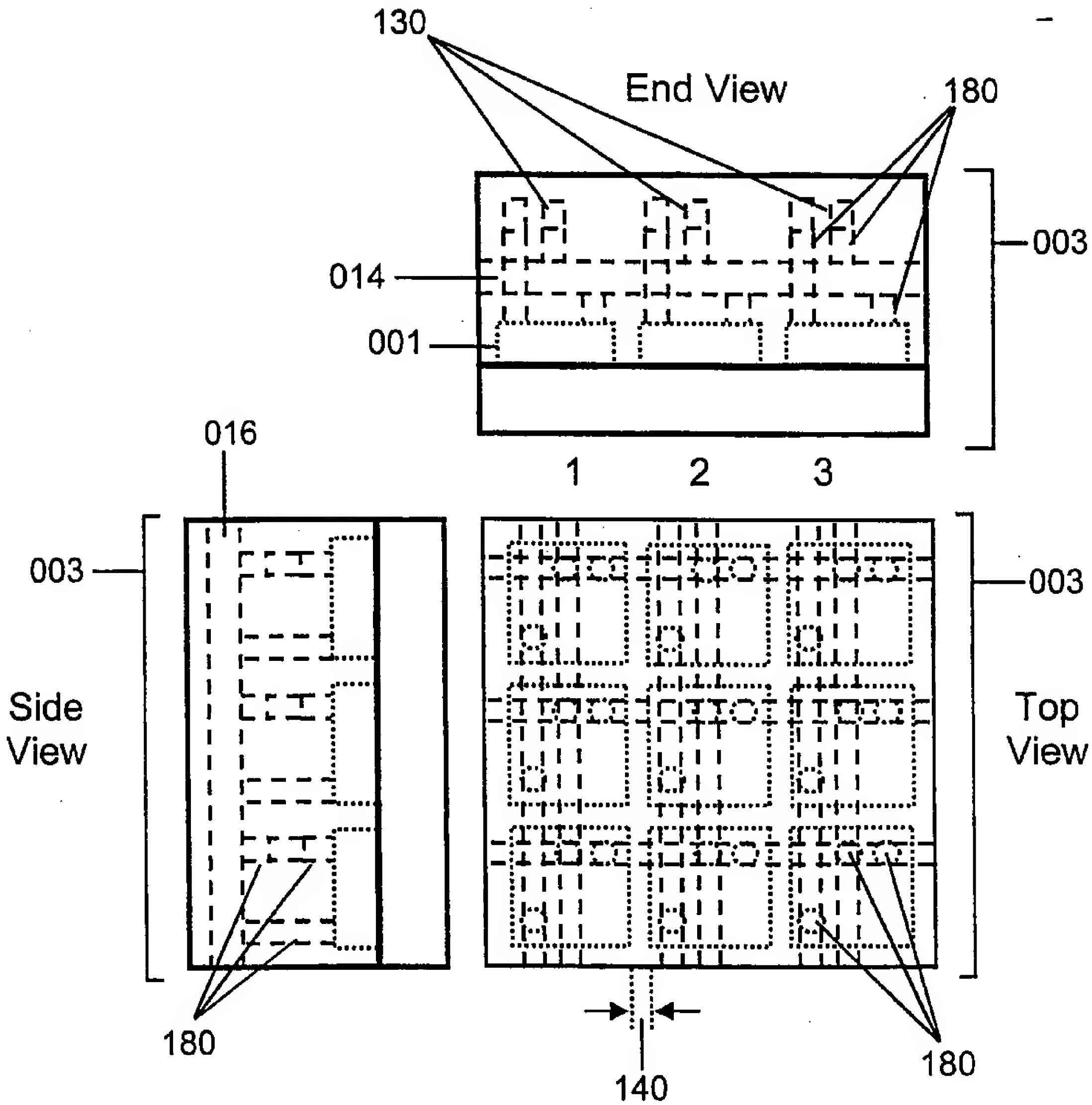

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Figure 35

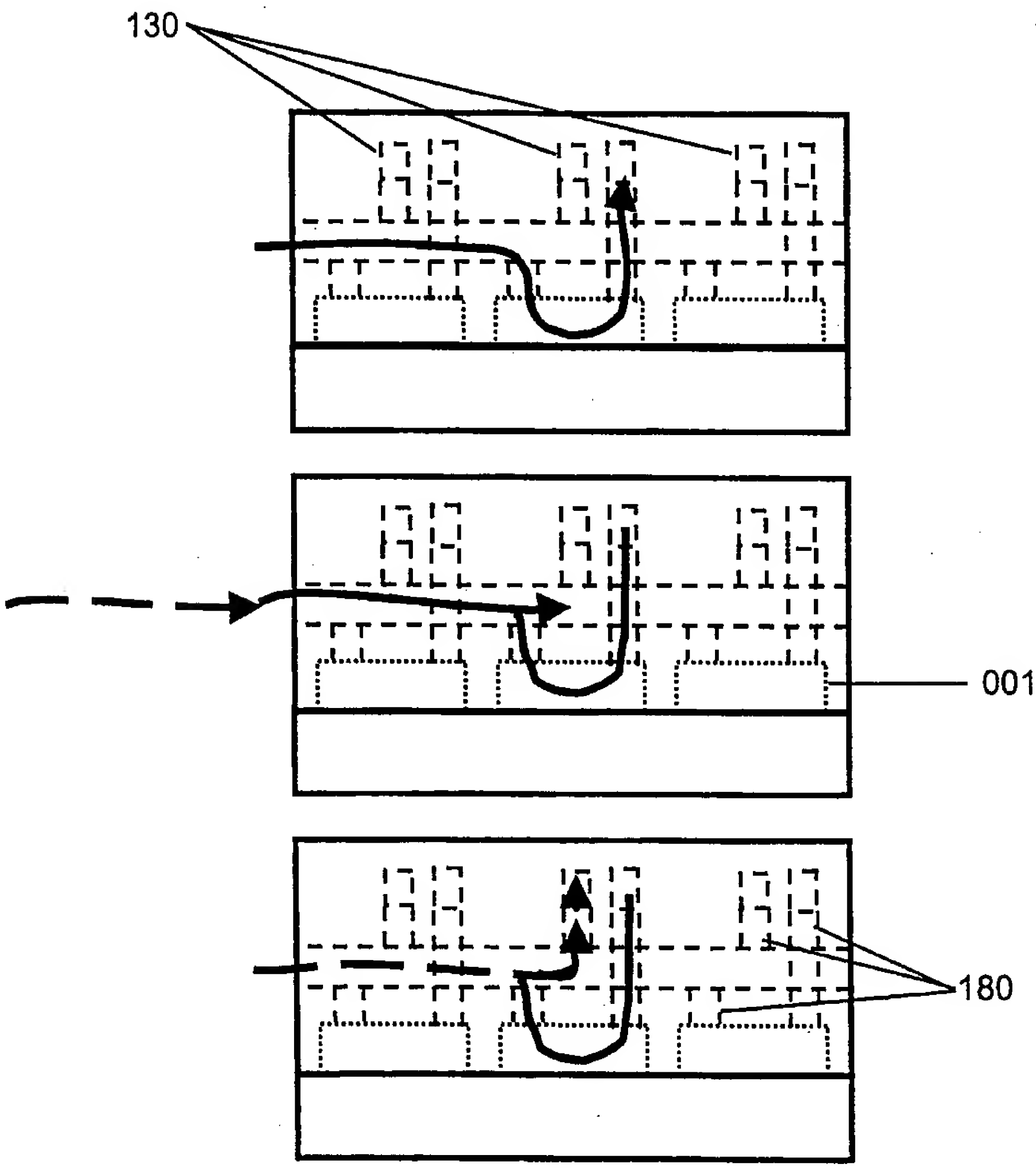

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Figure 36

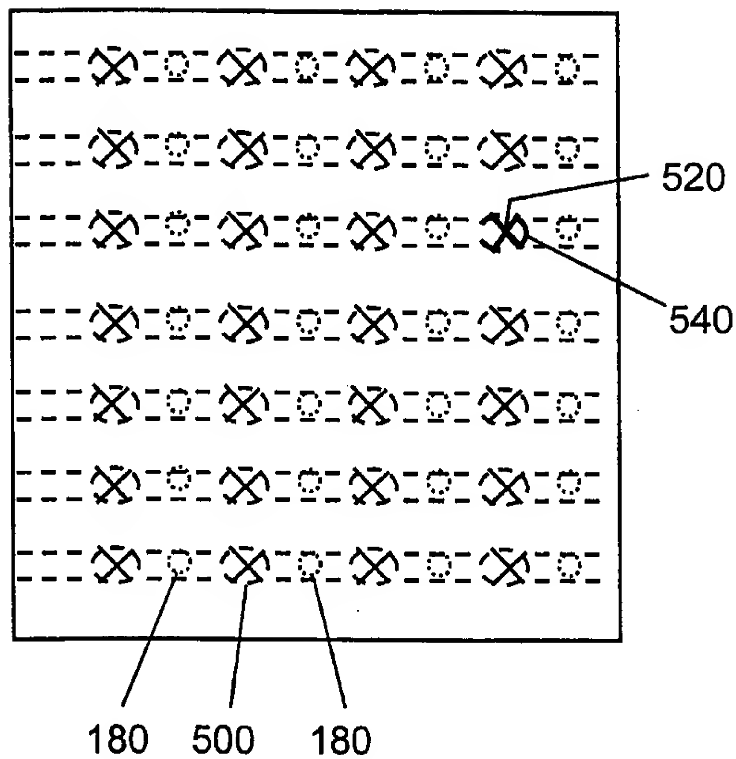

Figure 37

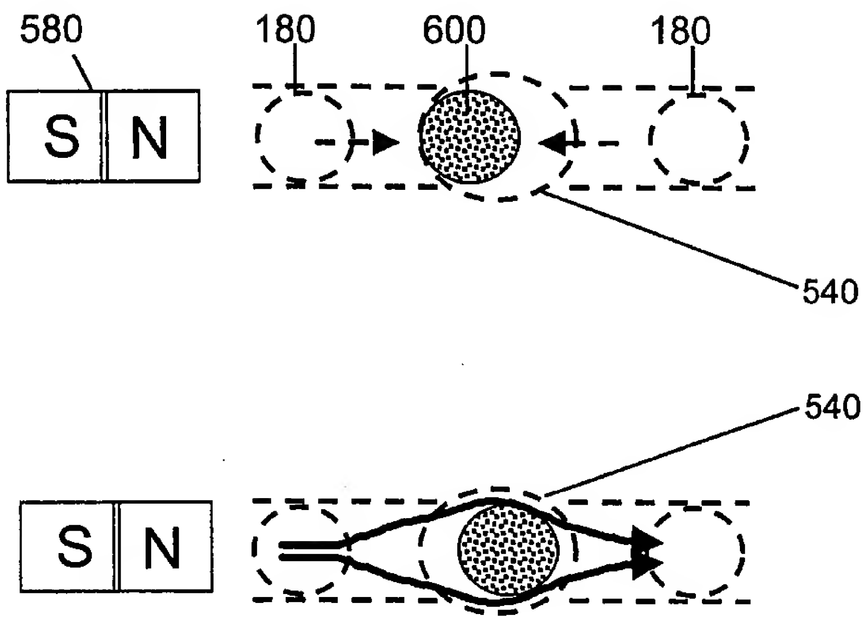

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Figure 38

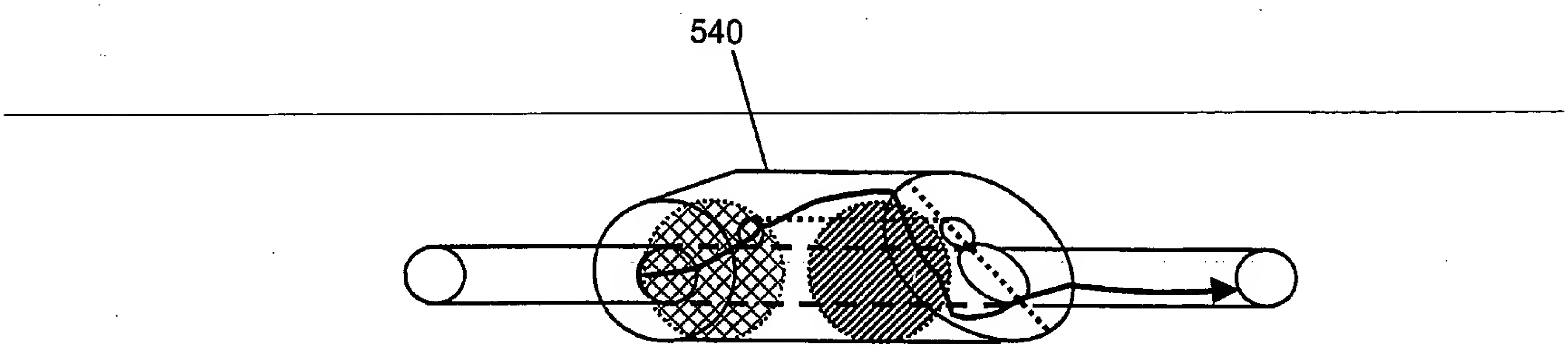

Figure 39

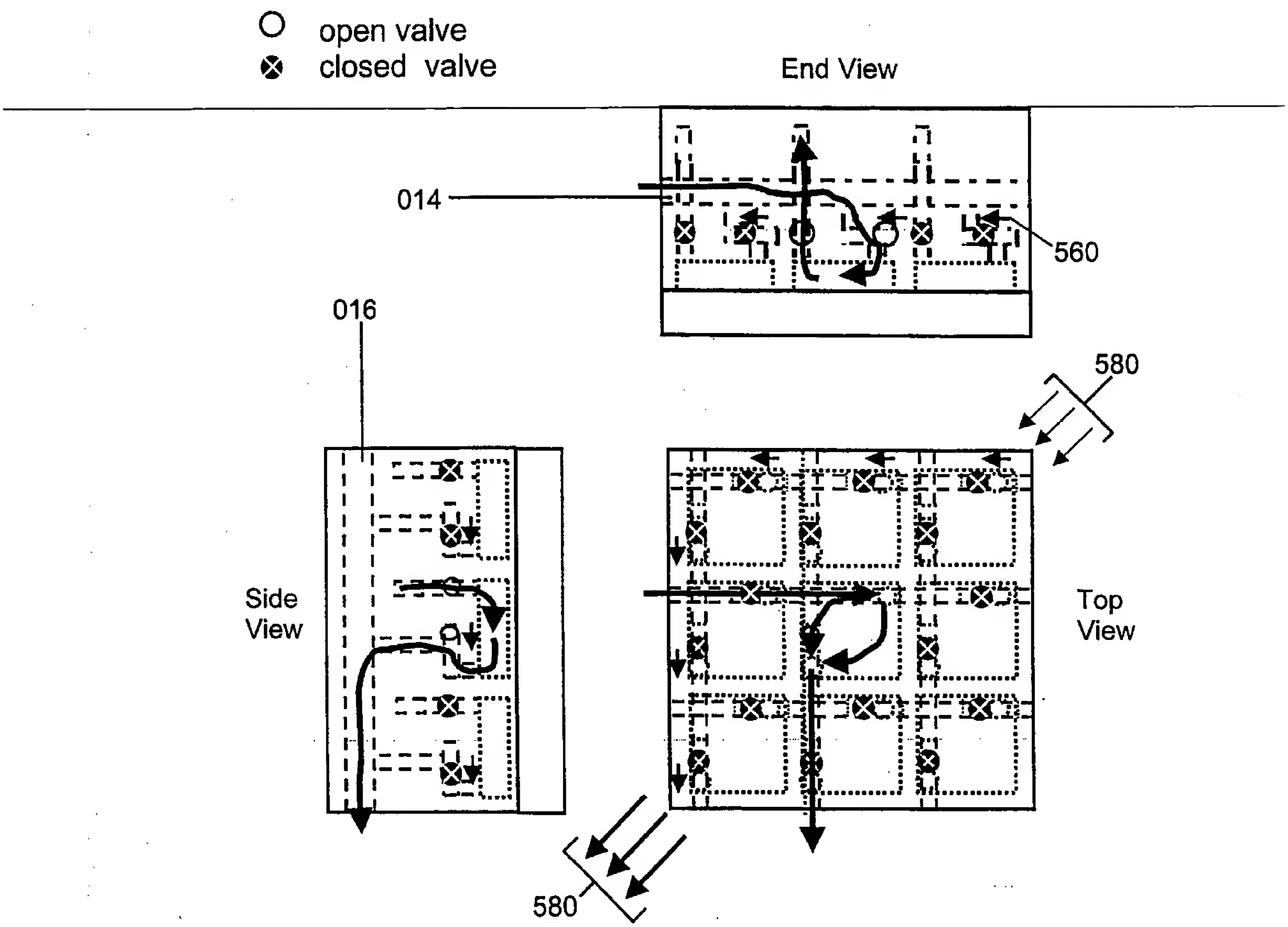
open valve
closed valve
End View
014
560
016
580
Side View
Top View
580

Figure 40

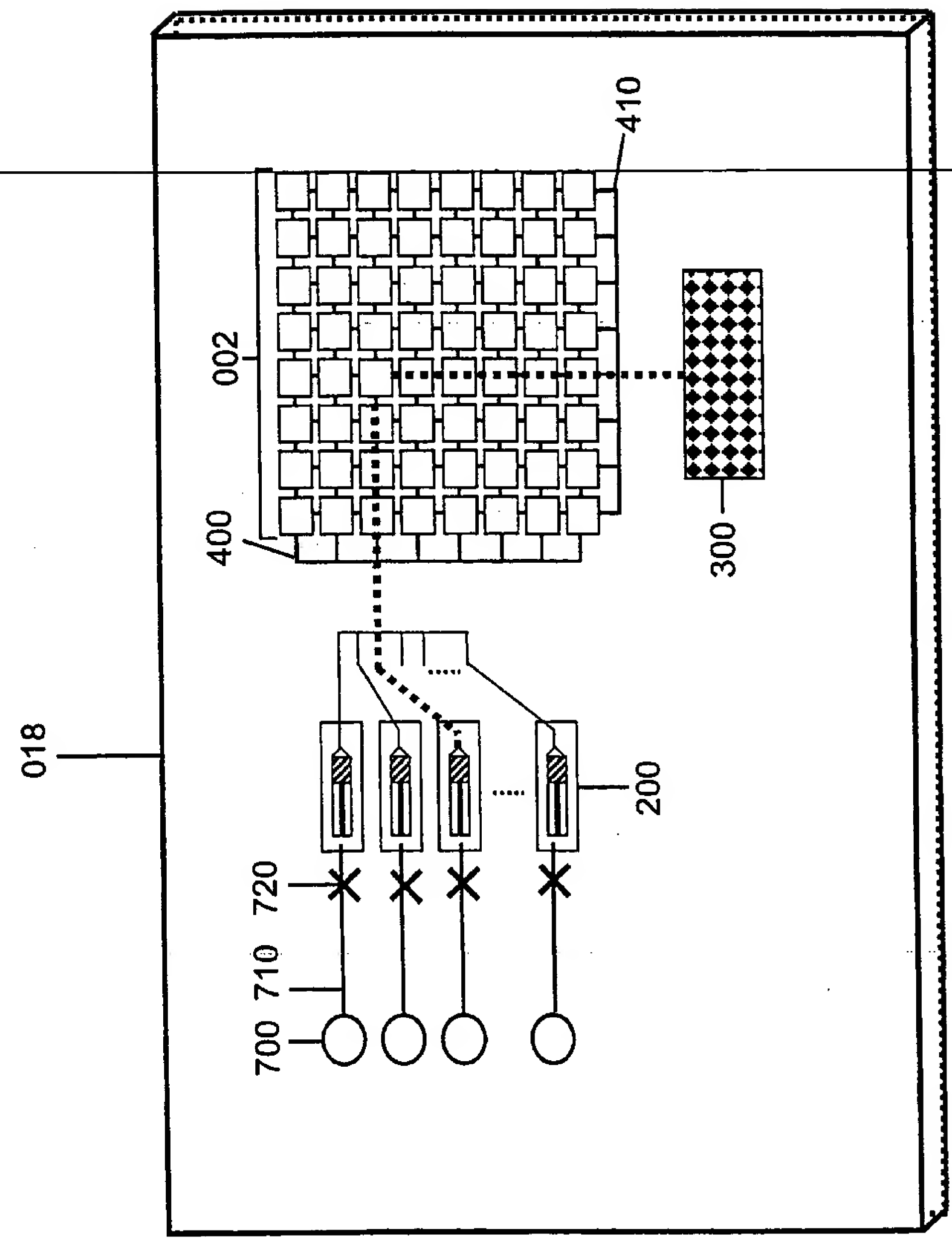

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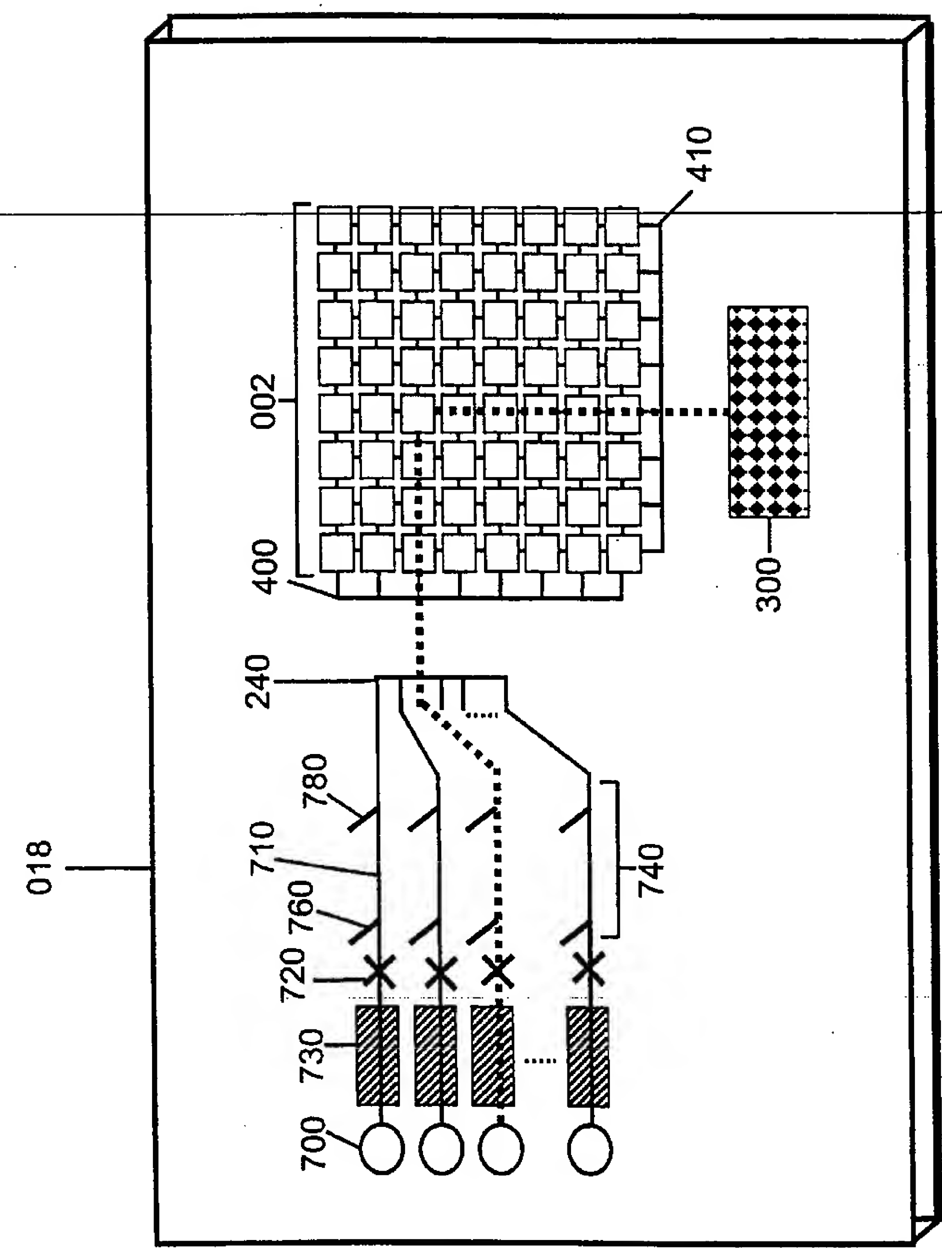
Figure 41



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